

Endothelial factors and platelet activity: Endothelin-1 a new modulator

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I declare that this thesis has been composed by me, and that the work described in it was carried out by me with the exception of acknowledged contributions executed under my direction.

I wish to dedicate this thesis to my family from the smallest to the tallest.

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## ABSTRACT

Endothelin-1 (ET-1) is a powerful vasoactive peptide. First isolated in 1988, ET-1 is both a vasoconstrictor, acting directly on vascular smooth muscle cells, and potentially a vasodilator acting on endothelial cells to cause the synthesis and release of endothelium derived mediators. The cellular responses to ET-1 are mediated by at least two receptors, the ET<sub>A</sub> and the ET<sub>B</sub> receptor. Early findings on the actions of ET-1 on blood platelets were inconclusive. It was, therefore the aim of the work presented in this thesis to investigate and characterise the effects of ET-1 on human platelets and to examine whether these effect were altered in subjects with essential hypertension.

To investigate the direct actions of ET-1 on human platelets, aggregation studies were performed in platelet rich plasma. Light transmittance platelet aggregometry was used to examine the effect of ET-1 alone and the effect of pre-incubation with ET-1 on adrenaline- and ADP -induced primary and secondary aggregation. ET-1 (1 $\mu$ M) alone caused slight but significant platelet aggregation ( $p=0.04$ ,  $n=14$ ). In addition ET-1 (1nM) significantly potentiated adrenaline induced-primary aggregation ( $p=0.04$ ,  $n=14$ ) and inhibited adrenaline-induced secondary aggregation ( $p=0.04$ ,  $n=14$ ). ET-1 did not significantly alter ADP-induced primary or secondary aggregation. It was concluded from these studies that ET-1 at high concentrations had a slight aggregatory effect and at lower concentrations modulated primary and secondary aggregation to adrenaline in a bi-directional manner.

The ET receptor subtype which mediated the potentiation of the pro-aggregatory actions of ET-1 on adrenaline-induced primary aggregation was investigated. Both the selective ET<sub>A</sub>/ET<sub>B</sub> agonist ET-1 and the ET<sub>B</sub> selective agonist SRTX S6c were employed as well as the ET<sub>A</sub> and the ET<sub>B</sub> selective antagonists BQ 123 and BQ 788 respectively. ET-1 and SRTX S6c dose dependently potentiated adrenaline induced primary aggregation ( $p=0.03$  &  $p=0.05$  respectively  $n=12$  for both). Furthermore the

potentiation of aggregation was attenuated by BQ 788 ( $p=0.04$ ,  $n=6$ ) but unaltered by BQ 123. Hence, it was concluded that potentiation of adrenaline induced aggregation by ET-1 is dose dependent and is largely mediated by the ET<sub>B</sub> receptor.

To investigate possible second messengers involved in ET receptor mediated effects platelets cyclic AMP and cyclic GMP levels were measured after incubation with ET-1 and SRTX S6c. Both ET-1 and SRTX S6c caused an increase in platelet cyclic GMP levels ( $p=0.03$  &  $p=0.01$  respectively,  $n=6$  for both). However neither agonists altered platelet cyclic AMP levels. From this it would appear that the platelet ET<sub>B</sub> receptor is associated with an accumulation of cyclic GMP but not cyclic AMP.

Additional evidence for the existence of specific binding sites for ET-1 on platelets was investigated by radio ligand binding studies and RT-PCR. RT-PCR was performed on RNA extracted from platelets isolated by centrifugation and on platelets isolated by centrifugation on discontinuous gradients of percoll. Mean binding of <sup>125</sup>I-ET-1 to platelet membranes was not significantly altered by increasing concentrations of cold ligand ( $p=0.18$ ;  $n=6$ ). However, in platelet preparations from two subjects, binding of cold ET-1 was dose dependently reduced providing  $K_D$ s of 1.1 and 0.4 nM. RT-PCR on RNA extracted from platelet isolated by centrifugation alone was positive for ET<sub>B</sub> mRNA for all subjects tested, but there was an indication that this preparation may have been contaminated by other blood cells. There was no evidence for contamination in the platelets prepared by centrifugation through percoll. In this preparation 4 of the 6 samples were positive for ET<sub>B</sub> mRNA. Although neither study produced evidence for ET receptors on platelet from all subjects tested, the results of both studies did indicate that ET receptors exist on platelet from some subjects.

To examine the role of ET-1-mediated release of endothelium derived platelet modulators on the action of ET-1 on platelets, an *ex vivo* study was performed. ET-1 was infused into the brachial artery, at the end of the infusion venous blood was sampled and *in vitro* aggregation studies were carried out. In an attempt to establish the role of ET derived prostacyclin *ex vivo* studies were carried out in the presence and absence of aspirin. Forearm blood flow was measured during all *ex vivo* studies. *In vitro* studies were also carried out on the same subjects. In the *ex vivo* studies ET-1 infusion caused a significant reduction in blood flow (32.8% at 60 min). This effect was significantly increased after intravenous aspirin infusion (50.3% at 60 min). ET-1 infusion did not significantly alter *ex vivo* primary platelet aggregation to adrenaline or ADP, nor did it alter ADP-induced secondary aggregation. However, adrenaline induced secondary aggregation was significantly attenuated ( $67.1 \pm 6.6$  vs  $38.5 \pm 8.7$ ). A similar pattern of results was observed when a comparable concentration of ET-1 to that estimated to be achieved *in vivo* by the ET-1 infusion was used *in vitro* in the same subjects. These results indicate that the *ex vivo* inhibition of platelet aggregation by ET-1 is independent of endothelium derived mediators.

To investigate whether platelet sensitivity to ET receptor agonists is altered in subjects with high blood pressure two separate studies were carried out. In the first study platelet aggregation to adrenaline was studied in the presence and absence of ET-1 in patients with essential hypertension and age/sex matched controls. Platelet cyclic nucleotide responses to ET-1 and SRTX S6c were also studied in the same subjects. In the second study subjects involved in a '4 corner study' designed to investigate familial predisposition to hypertension were recruited. Subjects participating in this study had not developed hypertension but represented individuals identified as having either high or low blood pressure, in addition they were also classified according to parental blood pressure. This allowed subjects with

a predisposition to hypertension, on the basis of having raised blood pressure, to be distinguished from subjects with a familial predisposition to hypertension, that is subjects with raised blood pressure who also have parents with raised blood pressure. Platelet aggregation to adrenaline was studied in the presence and absence of ET-1.

In the first study there was no difference in the ET-1 mediated potentiation of adrenaline-induced primary aggregation between the two groups. However, although ET-1 inhibition of secondary aggregation was observed in the control group ( $p=0.001$ ,  $n=10$ ) this effect was not observed in the hypertensive subjects. ET-1 tended to increase cGMP in platelets from the control patients but this did not achieve statistical significance. SRTX S6c did significantly stimulate cyclic GMP accumulation in platelets from the control subjects ( $p=0.04$ ,  $n=10$ ), but did not significantly alter cyclic GMP levels in platelets from the hypertensive subjects. Neither SRTX S6c nor ET-1 altered platelet cyclic AMP levels in either group. It would appear from this study that patients with established essential hypertension have a reduction in the anti-aggregatory response to ET-1 and may have impaired platelet cyclic GMP responses.

In subjects from the '4 corner' study there was a tendency for ET-1 to potentiate adrenaline-induced primary aggregation in all 4 corners. However, this effect was only significant in the corner representing subjects with high blood pressure who had parents with high blood pressure ( $p=0.04$ ,  $n=11$ ). ET-1 inhibition of secondary aggregation did not achieve statistical significance in any of the 4 corners, although a consistent tendency was observed in all 4 corners. The potentiation of adrenaline-induced primary aggregation by ET-1 may be associated with a familial predisposition to hypertension and therefore the progression of hypertension in these individuals may be associated with increased ET-1 sensitivity.

In conclusion, the work in this thesis presents evidence for ET-1 modulation of adrenaline mediated platelet aggregation *in vitro* and *ex vivo*. The receptor sub-type mediating the potentiation of adrenaline-induced primary aggregation by ET-1 appears to be the ET<sub>B</sub> receptor sub-type. In addition the ET<sub>B</sub> receptor appears to mediate an increase in platelet cyclic GMP levels. Both the inhibition of aggregation and the stimulation of cyclic GMP by ET receptor agonists is altered in platelets from patients with essential hypertension, which might contribute to the pro-aggregatory state of platelets from such patients. This would suggest that the actions of ET-1 could be a possible site for pharmacological intervention to prevent some of the consequences of hyperactive platelets in hypertensive patients. Finally, the fact that the potentiation of primary aggregation by ET-1 might be associated with a familial predisposition to hypertension would suggest that in some individuals the development of hypertension may be related to an increase sensitivity of the ET<sub>B</sub> receptor.



## **PUBLICATIONS ARISING FROM THIS THESIS**

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## **LIST OF ABBREVIATIONS FREQUENTLY USED**

5HT	Serotonin
AA	Arachidonic acid
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
[Ca <sup>2+</sup> ] <sub>i</sub>	Intracellular calcium ion concentration
cyclic AMP	Adenosine 3',5'-cyclic monophosphate
cAMP-PK	cAMP dependent protein kinases
cyclic GMP	Guanosine 3',5'-cyclic monophosphate
cGMP-PK	cGMP dependent protein kinase
CICR	Calcium induced calcium release
DAG	Diacylglycerol
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Nucleotide triphosphate
DTT	Dithiothreitol
ECE	Endothelin converting enzyme
EDRF	Endothelium derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
ET	Endothelin
HR	Heart rate
I(1,4,5)P <sub>3</sub>	Inositol (1,4,5) trisphosphate
ir-ET-1	Immunoreactive endothelin-1
K <sub>D</sub>	Dissociation constant
LNMA	L- <i>N</i> <sup>G</sup> -monomethyl arginine
MAP	Mean arterial pressure
MMLV	Moloney murine leukaemia virus reverse transcriptase

NO	Nitric oxide
NOS	Nitric oxide synthase
PG	Prostaglandin
PIP <sub>2</sub>	Phosphatidylinositol 4,5-bisphosphate
PMSF	Phenylmethylsulphonylfluoride
PPP	Platelet poor plasma
PRP	Platelet rich plasma
RIA	Radio immunoassay
RNA	Ribonucleic acid
RT PCR	Reverse transcriptase polymerase chain reaction
SAH	Sub arachnoid haemorrhage
SccAMP TME	Succinyl cyclic AMP tyrosinemethylester
SccGMP TME	Succinyl cyclic GMP tyrosinemethylester
s.e. mean	Standard error of the mean
SNP	Sodium nitroprusside
SRTX	Sarafotoxin
TxA <sub>2</sub>	Thromboxane A <sub>2</sub>
VASP	Vasodilator stimulated phosphoprotein,

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## **CHAPTER 1**

### **INTRODUCTION**

The vascular endothelium is involved in mediating the constriction and relaxation of the underlying smooth muscle by the release of a number of agents, such as nitric oxide (NO) and ET-1. The factors may be released basally or when activated by physical and chemical stimuli. By regulating the lumen size of the vessels the endothelium is able to modulate local blood flow. Many endothelium-derived factors are released both lumenally and ablumenally. In the former case they may act to regulate blood platelet activity. In healthy vessels the expected role of the endothelium may be the prevention of unnecessary platelet aggregation and thrombus formation. However, at sites of vascular injury the inhibitory role of the endothelium on platelet aggregation may be modified to allow platelets access to the site of damage to commence repair. In many cardiovascular conditions the platelet-endothelium interaction may be impaired, thus facilitating thrombus formation, further vessel constriction and consequently ischaemic damage. The key regulatory role of the endothelium on platelet function and the prevalence of cardiovascular disease in Western society has made the topic of platelet-endothelium interaction an area of increasing interest.

### The Platelet

Platelets are cell fragments which circulate in the vascular system at counts of between  $150-400 \times 10^9/L$ , in the healthy adult human. With an approximate mean diameter of 2-3  $\mu m$  and thickness of 1  $\mu m$  platelets are the smallest blood 'cells'. Highly reactive to external stimuli, the principal function of platelets is to ensure haemostasis. In response to exposure of sub-endothelial collagen, indicating a disruption of the lining of the vasculature, platelets may, adhere to the site of damage, form aggregates and release some of the contents of platelet storage granules. These responses, the stimuli that provoke them, and some of the intra-platelet biochemical signalling that mediate them, will be discussed before

examining the putative role of the powerful vasoconstrictor peptide ET in the regulation of platelet activity. The anucleate status of platelets means that they are not strictly speaking cells, but the similarity of much of their biochemistry allows them often to be referred to as such and hence in this thesis they will be referred to as cells and their attributes as cellular.

Platelets are derived from megakaryocytes in the bone marrow. As early as 1943 (Jappa, 1943) megakaryocytes were identified as having nuclear lobes and it was concluded that during maturation the megakaryocyte increases its' nuclear material without undergoing cell division. The significance of the differing number of nuclear lobes of mature megakaryocytes on the reactivity of the resulting platelets has become a topic of some interest (Kristensen & Martin, 1991). Mature megakaryocytes become amoeboid and develop pseudopodia which enter into the circulation through the marrow sinusoids. Some pseudopodia break off to form proplatelets, but whole megakaryocytes also enter the circulation. Proplatelets and megakaryocytes remain in the pulmonary circulation until they fragment to produce discoid platelets (Kaufman *et al.*, 1965). The production of platelets from megakaryocytes might indicate that regulation of the circulating platelet number depends completely on an alteration in megakaryocyte maturation or egress into the circulation. However, a more rapid form of control is afforded by the fact that approximately one third of platelets are stored in the spleen, as an interchangeable reservoir with platelets in circulation.

Platelets are described as having four zones, which relate the structure of the platelet to the functions. The peripheral zone consists of the glycoprotein-rich outer membrane and submembrane filaments. This outer membrane, although differing in thickness and precise constituents, is similar to that of other cell types consisting of a lipid bilayer containing a variety of receptors and ion pumps. The sol-gel zone

contains the cytoplasmic matrix of the platelet. The various filaments of the sol-gel zone are responsible for the maintenance of the quiescent discoid shape of the platelet and, on polymerisation of actin proteins, for the characteristic shape change induced by most platelet activating agents. The organelle zone contains mitochondria and ribosomes as well as the three types of platelet granules; lysosomes, dense bodies and alpha granules. The three types of granules have distinct functions: the lysosomes contain degradative enzymes; the dense bodies contain serotonin, ADP and calcium involved in platelet aggregation and maintenance of vessel tone; and the alpha granules contain platelet derived growth factor, platelet factor 4 and  $\beta$ -thromboglobulin, the last often being used as an indicator of *in vivo* platelet activation. The fourth region is the membrane system. Two membrane systems exist; the open canalicular system (sometimes referred to as the surface connecting system) and the dense tubular system. The canalicular system effectively increases the surface area of the platelet exposed to the plasma. The dense tubular system interconnects with the canalicular system and appears to be a storage site for calcium. In addition the dense tubular system may also be a site for platelet prostanoid production (Blockmans *et al.*, 1995).

### Platelet Function

The highly reactive nature of platelets is integral to the function of the platelet as a prompt responder to vascular injury, and the diverse nature of the agents that stimulate platelet activity indicate the complexity of the stimuli which platelets can respond to. Stimulators of platelet activation include stimuli on the luminal surface of the blood vessels such as the structural protein collagen, as well as a number of humoral agents including thrombin, a proteolytic enzyme generated by the coagulation system; ADP and serotonin, liberated from activated platelets, and the catecholamine adrenaline and the peptide vasopressin, both of which are present in



the circulation. These diverse agents all contribute to a common endpoint platelet activation. However, the process leading to activation can be identified in a number of distinct but related stages.

The stages of platelet activation following stimulation are adhesion, shape change, primary aggregation, secretion and secondary aggregation. On exposure to denuded blood vessels the first measurable response of platelets is to adhere to the exposed sub-endothelial material and form a monolayer of platelets (Sixma *et al.*, 1987). Two of the best understood stimulators of platelet adhesion are collagen and von Willebrand Factor which form physical links to glycoproteins on the platelet surface. Platelet shape change follows adhesion or, for platelets in suspension, is itself the first stage in platelet activation. On stimulation by low concentrations of most aggregating agents, platelets change from discoid shape to spheroid and form pseudopodia. The formation of pseudopodia greatly increases the surface area of the platelet, facilitating further chemical and physical interactions. Platelet shape change is an event distinct from aggregation, not fully induced by adrenaline or phorbol ester (Affolter & Pletscher, 1982). It is not a prerequisite for aggregation and in the case of ADP shape change is probably mediated by a separate receptor to that mediating the aggregatory response (Colman, 1990). However, although a useful indication of low level platelet activation *in vitro*, the precise physiological function of shape change without aggregation is unclear.

*In vitro* analysis of platelet aggregation has identified two distinct phases of aggregation termed primary and secondary aggregation. Primary aggregation can be a reversible event which can be quantified both by light transmittance aggregometry (Born, 1962) and by measuring the disappearance of single platelets from a suspension (Reuter & Deter, 1979). The key event in primary aggregation is the exposure or activation of the fibrinogen receptor (the GPIIb/IIIa molecule) on the

cell surface. The symmetrical structure of fibrinogen allows the platelets that come into contact to stick together by the formation of fibrinogen bridges. The precise trigger that converts GPIIb/IIIa into the active state is not known. However, the principal mechanism appears to be calcium dependent. Activation of a protein kinase C in the absence of calcium does induce primary aggregation, but it tends to be much slower than calcium dependent aggregation. Primary aggregation, like shape change, appears to represent a functional response which is directly dependent on platelet aggregating agent receptor activation.

Secondary aggregation is associated with platelet granule release. Granule release is brought about by a synergistic combination of the actions of a protein kinase C and increased intra-platelet calcium levels which facilitate the fusion of the granule membranes with either the plasma membrane or the membrane of the open canalicular system (Walker & Watson, 1993). Although a protein kinase C independent mechanism for granule release has been proposed (Hashimoto *et al.*, 1994) the nature of such a mechanism is not known. 'Weak' agonists, such as ADP and adrenaline induce granule release and, hence, secondary aggregation in a manner dependent on amplification of the primary aggregation stimulus. This mechanism is largely facilitated by the activation of the calcium dependent phospholipase A<sub>2</sub> and the consequent production of thromboxane A<sub>2</sub> (Mustard *et al.*, 1975). 'Strong' agonists such as thrombin are able to elicit granule release without primary aggregation. The release of dense granule contents, including ADP, ATP, serotonin, adrenaline and calcium, stimulates further platelet aggregation, while the alpha granule contents, such as thrombospondin and fibrinogen are responsible for the formation of large, stable, irreversible aggregates. The secondary phase of aggregation observed *in vitro*, in the presence of a limited number of platelets, is thought to represent the formation of large stable aggregates rather than the creation of more aggregates (Huang & Detwiler, 1987). It is, therefore, clear that the

difference between primary and secondary aggregation is qualitative rather than quantitative.

All the known endogenous agents that regulate platelet activity, both stimulatory and inhibitory, are thought to exert their effects via receptors on the platelet surface membrane, with the important exception of NO which is discussed later. The two main receptor types are glycoproteins and guanine nucleotide binding regulatory protein linked receptors (G protein linked receptors). The principal glycoprotein activators are collagen, von Willebrand factor and fibrinogen. Of these, collagen appears to be the only one that significantly stimulates intraplatelet second messenger activity, von Willebrand factor and fibrinogen acting primarily as physical linkages. All of the stimulatory G protein linked receptors identified to date are polypeptides, consisting of an extracellular N terminal, seven transmembrane domains and an intracellular C terminal, which is responsible for second messenger generation.

Consideration of the receptors for thrombin, serotonin and adrenaline will be used to illustrate some of the nature of the pro-aggregatory G protein linked receptors. Thrombin is a powerful platelet stimulus causing shape change, aggregation and release of all three types of platelet granule. Thrombin activates its receptor by cutting off a portion of the N terminal of the receptor leaving the remaining N terminal capable of activating the receptor (Vu *et al.*, 1991). Thrombin receptor occupancy stimulates phospholipase C activation leading to an increase in intracellular calcium.

Serotonin is a much weaker platelet stimulus, capable of inducing shape change and reversible aggregation (Baumgartner & Born, 1968). The platelet serotonin receptor is of the 5HT<sub>2</sub> type, and consequently its actions can be blocked by ketanserin (De

Clerck *et al.*, 1982). Although serotonin alone is a weak platelet aggregating agent, released from platelet granules it is able to amplify the aggregation response elicited by other agonists. This potentiation depends on the time interval of exposure of the platelets to the separate agents (De Clerck & Vanhoutte, 1982). Like thrombin, serotonin activation of platelets is associated with increased phospholipase C activity.

Adrenaline is also termed a 'weak' platelet activating stimulus, it stimulates primary aggregation but does not stimulate secondary aggregation uniformly in platelets from all human donors (Arkel *et al.*, 1977). Adrenaline does not cause platelet shape change. The ability of adrenaline to induce platelet aggregation in the absence of other platelet aggregating agents remains a point of debate (Nieuwland *et al.*, 1993; Siess, 1989; Steen *et al.*, 1993). However, Steen *et al* concentrated on the failure of adrenaline to stimulate phospholipase C activity, whilst Nieuwland provided evidence for a direct  $\alpha_2$  adrenergic receptor mediated stimulation of protein kinase C. The pro-aggregatory effects of adrenaline are indeed mediated by the  $\alpha_2$  adrenergic receptor. Adrenaline stimulation of the  $\alpha_2$  receptor both promotes protein kinase C activation, by a phospholipase C independent mechanism, and inhibits adenylate cyclase. It has been suggested that  $\alpha_1$  receptors linked to phospholipase C may be present on platelets in approximately 30% of humans (Grant & Scrutton, 1979). In addition to the pro-aggregatory intracellular signalling described, thrombin and adrenaline are also able to increase adenylate cyclase activity, which is an inhibitory signalling mechanism. In the case of thrombin, adenylate cyclase activity is thought to be mediated by the same receptor that is linked to increase phospholipase C activity, but in the case of adrenaline,  $\beta_2$  adrenergic receptors are coupled to adenylate cyclase activation.

Prostaglandins (PG)  $E_1$  and  $I_2$  also act on platelets through a G protein linked receptor. However, these prostaglandins have an inhibitory effect on platelet aggregation. Both agonists act via a  $PGI_2$  receptor, which stimulates adenylate cyclase activity and increases platelet cyclic AMP levels. As mentioned above, NO also inhibits platelet activation (Radomski *et al.*, 1987). In contrast to the various agonists described above, NO does not act on a cell surface receptor. Instead, uniquely, NO diffuses across the cell membrane and directly stimulates soluble guanylate cyclase, and thus inhibits platelet aggregation. The recognition of the existence of calcium dependent constitutive nitric oxide synthase in platelets (Radomski *et al.*, 1990) suggests a parallel for the role for NO in platelet as in endothelial cells, as an intracellular as well as an extracellular messenger.

### Platelet Biochemistry

Intracellular signalling in platelets is regulated at many levels by some parallel and some interacting biochemical pathways. This is further complicated by the existence of several subtypes of the various key enzymes involved such as; protein kinase C,  $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\zeta$  (Baldassare *et al.*, 1992), phospholipase C,  $\beta$ ,  $\gamma$  and  $\delta$  (Berridge, 1993), and calcium dependent and 'independent' phospholipase  $A_2$  (Murthy *et al.*, 1995). The following discourse, therefore, is not intended to be a comprehensive description of the messengers mediating receptor activation, but an introduction to those most relevant to this thesis.

Three families of phospholipase enzymes play an important role in mediating intracellular events in platelets: phospholipase C, phospholipase  $A_2$  and phospholipase D. Phospholipase C is directly activated by a number of G protein linked receptors, and is one of the major pathways of platelet activation. Phospholipase C hydrolyses phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ) to form



inositol trisphosphate (I(1,4,5)P<sub>3</sub>) and diacylglycerol (DAG). I(1,4,5)P<sub>3</sub> elicits a rapid transient rise in intracellular calcium, [Ca<sup>2+</sup>]<sub>i</sub>, by acting on an IP<sub>3</sub> receptor on the dense tubular system and releasing calcium from intracellular stores. DAG, the other product of PIP<sub>2</sub> hydrolysis, is associated with the translocation and activation of some sub-types of protein kinase C (Baldassare, *et al.*, 1992). Platelet aggregation induced by DAG, and its synthetic derivatives such as phorbol esters, is mediated by protein kinase C activation. Phospholipase A<sub>2</sub> is involved in the early stages of prostaglandin and hence thromboxane A<sub>2</sub> (TxA<sub>2</sub>) synthesis. Phospholipase A<sub>2</sub> liberates arachidonic acid (AA) from phosphatidyl choline and phosphatidyl ethanolamine. AA is subsequently converted by cyclooxygenase to prostaglandins G<sub>2</sub> and H<sub>2</sub>. PGH<sub>2</sub> is converted in turn by thromboxane synthase to TxA<sub>2</sub>. PGG<sub>2</sub>, PGH<sub>2</sub> and TxA<sub>2</sub> are all potent, but labile aggregating agents able to cause shape change, primary aggregation and secondary aggregation (Armstrong *et al.*, 1983; Siess *et al.*, 1983). Thrombin and collagen have been shown to activate phospholipase D in human platelets (Chiang, 1994). Although its precise role is not clear there is some evidence that the main product of phospholipase D, phosphatidic acid may be associated with a calcium independent granule secretory mechanism. Phospholipase D appears to be a secondary response, at least partially dependent on protein kinase C activation (Chiang, 1994).

Protein kinase C has the potential for bi-directional regulation of platelet activity. It plays an important role in promoting both platelet adhesion and granular secretion and, conversely, protein kinase C has inhibitory effects on platelet function, inhibiting IP<sub>3</sub> formation and promoting calcium extrusion (Pollock *et al.*, 1987; Watson & Lapetina, 1985). As stated above, one of the main pathways for protein kinase C activation is the phospholipase C mediated production of DAG but other mechanisms do exist. Recently, Nieuwland described an α<sub>2</sub> adrenergic receptor G-protein linked activation of protein kinase C (Nieuwland, *et al.*, 1993) and Kroll

described protein kinase C activation by platelets subjected to pathological levels of shear stress without DAG formation (Kroll *et al.*, 1993). At least two targets exist for phosphorylation by protein kinase C, the 47 kDa protein pleckstrin and myosin light chain, although the precise function of the former remains obscure, the latter is involved in the contractile system in platelets (Watson *et al.*, 1993).

Although protein kinase C can act synergistically with calcium it can also exert effects in a calcium independent fashion. Recent work has shown that protein kinase C translocation occurs in two stages, an early transient stage and a sustained stage (Hashimoto, *et al.*, 1994), and that the early phase is not calcium dependent. The initial phase appears sufficient to elicit maximal serotonin secretion from platelets.

Although there is a potential role for protein kinase C as an inhibitor of platelet aggregation, the main intracellular messengers associated with this role are the cyclic nucleotides, cyclic AMP and cyclic GMP. The predominant regulation of cyclic AMP levels is by G protein activation of membrane bound adenylate cyclase, converting ATP to cyclic AMP, and degradation of cyclic AMP by a phosphodiesterase. Stimulation of cyclic AMP dependent protein kinases, cAMP-PK, types I and IIb, and the consequent phosphorylation mediate the diverse effects of cyclic AMP (Eigenthaler *et al.*, 1992). There appear to be several substrates for cAMP-PK including vasodilator stimulated phosphoprotein, (VASP), and myosin light chain kinase, phosphorylation of the latter by cAMP-PK prevent its activation by calcium. Platelet cyclic GMP levels are regulated predominantly by soluble guanylyl cyclase activation by NO, either as an intercellular or extracellular messenger, and degradation by a phosphodiesterase. There is recent evidence for a non-NO mediated increase in platelet cyclic GMP (Shen & Hong, 1995). Cyclic GMP has two main targets, cyclic GMP dependent protein kinase (cGMP-PK) and the cyclic GMP inhibited phosphodiesterase. The latter allows cyclic GMP to

indirectly regulate cyclic AMP levels, by decreasing the phosphodiesterase activity, and may to some extent account for the synergism observed with the actions of the cyclic AMP mediated PGI<sub>2</sub> and the cyclic GMP mediated NO in human platelets (Radomski, *et al.*, 1987). The only substrate so far identified for cGMP-PK in platelets is VASP. It would seem more than possible that VASP might mediate the inhibition of phospholipase C and the consequent reduction in protein kinase C activity observed as a result of activation of cAMP-PK and cGMP-PK (Waldmann & Walter, 1989). However, whether VASP is involved in activation of intracellular calcium pumps by cyclic AMP and cyclic GMP or not is less clear (Johansson & Haynes, 1992; Johansson *et al.*, 1992; Waldmann & Walter, 1989)

Much of the intracellular regulation of platelet activity described above is involved in the control of available intracellular calcium; the increase in  $[Ca^{2+}]_i$  through I(1,4,5)P<sub>3</sub> and the decrease by cyclic AMP and cyclic GMP. Activation of pro-aggregatory platelet receptors results either directly or indirectly in a rise in  $[Ca^{2+}]_i$ , and elevation of  $[Ca^{2+}]_i$  without receptor occupancy by A23187 also results in platelet activation (White *et al.*, 1974). Elevation of  $[Ca^{2+}]_i$  can be brought about by either the release of calcium from intraplatelet stores in the dense tubular system or calcium entry from the extracellular medium. The role of I(1,4,5)P<sub>3</sub> in the release of calcium from intracellular store has been considered above. I(1,4,5)P<sub>3</sub> independent pathways of calcium release are less well characterised. Calcium induced calcium release (CICR) has been described in a number of cells and may be partially responsible for the complex nature of calcium signalling (Berridge, 1991). Initiation of this system is dependent on I(1,4,5)P<sub>3</sub> and is followed by a CICR mechanism for propagation of the signal. A similar model has been proposed for platelets (Tsunoda *et al.*, 1988). Calcium entry may be brought about by receptor operated channels or intracellular calcium store regulated entry; it is thought that voltage operated calcium channels do not exist in platelets (Rink & Sage, 1990) and there is little evidence for



second messenger operated channels. ADP had been shown to activate receptor operated calcium channels in platelets, calcium entry which occurred without measurable delay was evinced to be through a receptor operated channel by patch clamp experiments (MahautSmith *et al.*, 1990). However, to date, no other agonist has been shown to activate such channels. Prevention of calcium re-uptake into intracellular stores by the use of thapsigargin prior to the exposure of the platelet to thrombin, has been used to demonstrate that store depletion leads to calcium entry (Sargeant *et al.*, 1992).

### Platelet activity in health and disease

As stated above one of the primary functions of platelets is the formation of a haemostatic plug, by the initial formation of the 'white thrombus', in response to endothelial damage and the consequent prevention of bleeding. This is followed by the release of platelet growth factors and repair of the damaged area. Many disorders associated with abnormal platelet function involve reduced platelet activity and prolonged bleeding. Prolonged bleeding may be associated with reduced platelet number (thrombocytopenia), due to impaired platelet production or increased destruction of platelets, or by conditions associated with ineffective coagulation, due to the absence of specific clotting factors, as seen in the various forms of haemophilia. The involvement of reduced platelet function in bleeding disorders is a complex haematological area but lies outside the remit of this thesis. It is the phenomenon of increased platelet activity and aggregation, often observed in cardiovascular disease, that will be considered below.

## Essential Hypertension

Platelet function in essential hypertension has been studied for a number of reasons. Platelets may play a role in the pathophysiology of hypertension by the release of vasoconstrictors such as serotonin from activated platelets and also as mediators of risks associated with hypertension. In addition, platelets are often described as a model for vascular smooth muscle cells due to the parallel control of platelets and vascular smooth muscle cells exerted by the vascular endothelium and the many receptors and intracellular pathways that they share, such as  $\alpha_2$  adrenergic and angiotensin II receptors, adenylate cyclase activity and calcium regulation.

A number of studies have suggested increased *in vitro* aggregation of platelets from patients with essential hypertension (Lechi *et al.*, 1989; Touyz & Schiffrin, 1993b; Vlachakis & Aledort, 1980). However, other groups have not observed any significant differences *in vitro* (Cadwgan & Benjamin, 1993; Mehta & Mehta, 1981). These apparent discrepancies may be due to the conditions under which these studies have been carried out and the population of hypertensive subjects studied. Measurements of indices of *in vivo* platelet activation do suggest a greater level of activity in hypertensives than controls (Islim *et al.*, 1992; Mehta & Mehta, 1981). Increased platelet calcium concentrations observed in hypertensive patients (Bühler & Resink, 1988; Islim, *et al.*, 1992; Le Quan Sang & Devynck, 1986; Mehta & Mehta, 1981) are consistent with the hypothesis that hypertension is associated with excess calcium mobilisation (Robinson, 1984). Other abnormalities observed in platelets from patients with essential hypertension include decreased NO synthesis after stimulation with collagen (Cadwgan & Benjamin, 1993) and lower platelet magnesium concentrations (Touyz & Schiffrin, 1993b), both traits may be associated with altered platelet function.

## Pre-eclampsia

Pre-eclampsia is a condition associated with hypertension in pregnancy. However, several aspects of this condition clearly distinguish it from transient hypertension including proteinuria and raised growth factor activity (Roberts & Redman, 1993). An increased arterial sensitivity to exogenous angiotensin II precedes the onset of hypertension (Brosens *et al.*, 1972). The combined factors of: an early reduction in platelet count in preeclampsia, increased platelet sensitivity to angiotensin II and; once again, the similarity of platelets to smooth muscle cells, led to the investigation of platelet function. Although basal platelet calcium levels are not altered, the arginine-vasopressin stimulated calcium response is significantly increased as early as the first trimester (Zemel *et al.*, 1990). Zemel highlights the fact that disturbances in platelet activity precedes those in vascular smooth muscle cells and consequently increased platelet activity may contribute to the pathogenesis of the disease.

## Ischaemic Heart Disease

Platelets activation is known to play an important role in the progression of a number of conditions associated with myocardial ischaemia such as angina and infarction (de Lorgeril *et al.*, 1994; Flores & Sheridan, 1994; Willerson *et al.*, 1989). Although the precise underlying causes of these conditions are not always fully understood it is known that vessel narrowing and occlusion are often part of the final pathway. Platelets may have two separate but associated functions, the formation of a thrombus and hence partial blockage of the vessel and the release of vasoconstrictor agents from aggregating platelets leading to a narrowing of the vessel. In response to a site of damage and the exposure of the subendothelial layer platelets will tend to aggregate as described earlier, but also in the case of localised dysfunction of the

endothelium platelets may also respond by forming aggregates. The efficacy of aspirin in the reduction of mortality in patients suffering myocardial infarction has been largely attributed to its ability to inhibit platelet cyclooxygenase .

Studies examining increased circulating platelet aggregates in patients suffering from unstable angina produced evidence for increased *in vivo* platelet aggregation coinciding with angina attacks (Dalal *et al.*, 1980; Robertson *et al.*, 1980). In a similar study where 44 patients were admitted to hospital with chest pain, the number of circulating platelet microthrombi were significantly increased in patients suffering from myocardial infarction but not in the patients without myocardial infarction (Mehta & Mehta, 1981). These studies, however, were not able to determine whether the platelets from patients suffering from ischaemic heart disease had a greater tendency to aggregate or whether aggregating agents were released during these attacks.

As part of the Caerphilly Collaborative study blood samples were taken from over 1800 subjects for platelet aggregation studies. Significant relations were shown between past myocardial infarction and ADP-induced *in vitro* platelet aggregation as well as significant relations between electrocardiographic evidence of ischaemia and ADP-induced aggregation (Elwood *et al.*, 1991). Interestingly, no such association was seen when collagen was used as the aggregating agent. A correlation between the rate of platelet aggregation to ADP and subsequent mortality from coronary heart disease was reported in a study in which 487 healthy middle aged men participated (Thaulow *et al.*, 1991), again no correlation was seen when collagen was the aggregating agent. A positive association was seen between *in vitro* platelet hyper-reactivity and both mortality and cardiac events in a group of survivors of myocardial infarction (Trip *et al.*, 1990). The fact that increased platelet reactivity is agonist specific in some of these studies suggests that there is a particular, but as yet

undefined, intraplatelet mechanism involved. The identification of such a mechanism may be of use in understanding the discrepancies between the results of some of the studies mentioned above.

The understanding of the actions of platelets in ischaemia is further complicated by the complex nature of the interactions between the vascular endothelium and platelets. The endothelium of arterioles possesses 5HT<sub>1</sub> receptors, which on exposure to serotonin released from platelets indirectly relax underlying vascular smooth muscle cells. In the absence of the endothelium serotonin caused constriction by acting directly on 5HT<sub>2</sub> receptors on the vascular smooth muscle cells (Vanhoutte, 1991). The endothelium not only has receptors for platelet derived agents, but is a source of vasoconstrictors and vasodilators which modulate platelet activity.

#### Platelets and the vascular endothelium.

The general structure of the vessel walls is fairly uniform throughout the circulatory system, with the exception of the capillaries, in that they consist of three separate layers. These are the tunica intima, tunica media and tunica adventitia. The tunica intima, the layer closest to the vessel lumen and hence the blood, consists of the endothelium and the basal lamina. The media is composed primarily of smooth muscle cells and collagen fibres, it is this layer that is absent in capillaries. The adventitia is made up of connective tissue and fibroblasts, and contains nerves and, in larger, vessels the vasa vasorum.

The endothelium is a continuous layer of single cells, lining the whole of the cardiovascular system. Up until the late 1970s the endothelium was simply seen as an anatomical barrier between the blood constituents and the vessel wall. However,

over the past twenty years it has come to be recognised as an important regulator of vascular tone and platelet activation.

The endothelium releases, both lumenally and ablumenally, a number of agents which regulate vascular smooth muscle tone and platelet activity respectively. In addition, the luminal surface of the endothelium contains factors that inhibit platelet aggregation, such as thrombomodulin and ADPase. Thrombomodulin binds thrombin, a strong platelet aggregating agent, making it inaccessible to platelets, while ADPase converts ADP to inactive AMP. These factors may be of great significance in the regulation of platelet aggregation. However, for the purpose of this thesis the following paragraphs will concentrate on factors released from the endothelium.

Prostacyclin was first discovered in 1976 (Moncada *et al.*, 1976). A member of the family of prostaglandins, it is synthesised from prostaglandin H<sub>2</sub> by the actions of the enzyme prostacyclin synthase. Its production is initiated by the actions of phospholipase A<sub>2</sub> which liberates arachidonic acid from membrane phospholipids. Arachidonic acid is converted to prostaglandin G<sub>2</sub> by cyclooxygenase, which in turn is converted to prostaglandin H<sub>2</sub>. The conversion of prostaglandin G<sub>2</sub> by cyclooxygenase is a common step in the production of both prostacyclin and thromboxane and is inhibited by aspirin, as stated above. The major metabolites of prostacyclin are stable derivatives of prostaglandin F<sub>2α</sub> which are assayed as indicators of prostacyclin production. Prostacyclin generation can be stimulated by both endogenous mediators and mechanical stimuli (Bhagyalakshmi & Freangos, 1989). Among the endogenous mediators are platelet derived serotonin and adenine nucleotides (Mügge *et al.*, 1991).



As with many endothelium derived mediators prostacyclin is a locally acting hormone; the ubiquity of the endothelium allows it to give a localised response to local stimuli. Acting via specific cell surface receptors, the intracellular action of prostacyclin is similar in both platelets and vascular smooth muscle cells, it stimulates adenylate cyclase to produce cyclic AMP, inhibiting platelet aggregation and relaxing vascular smooth muscle cells.

In 1980 Furchgott and Zawadzki identified another endothelium derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980). EDRF was known not to be a prostaglandin as its actions were not inhibited by cyclooxygenase blockade. Further investigation revealed the intracellular messenger for EDRF was cyclic GMP (Ignarro & Kadowitz, 1985); it had previously been established that NO stimulated cyclic GMP production (Greutter *et al.*, 1979). In addition the actions of EDRF were inhibited by haemoglobin (Martin *et al.*, 1985). Furthermore it had been known for some time that NO binds to the haeme moiety of haemoglobin (Kon, 1968). In 1987 Palmer and colleagues indirectly measured NO in an experiment where bradykinin, a substance known to release EDRF, was applied to strips of rabbit aorta (Palmer *et al.*, 1987). The amount of NO measured was calculated to account for the relaxation of the strips apparently induced by EDRF. It has since been widely accepted that EDRF is NO.

NO and L-citrulline are synthesised from the substrate L-arginine by the actions of the enzyme NO synthase (NOS) (Palmer *et al.*, 1988). NO production can be competitively inhibited by a number of substances with structural similarity to L-arginine, such as L- *N*<sup>G</sup>-monomethyl arginine (LNMMA). To date, three distinct isoforms of NOS have been identified; two constitutive calcium dependent isoforms found in the cardiovascular system and the nervous system (eNOS and nNOS respectively) and an inducible calcium independent isoform, widely distributed and

expressed during inflammatory reactions (iNOS). Generation of NO by eNOS can be stimulated by an increase in intracellular calcium (Schulz & Triggle, 1994). NO freely diffuses into adjacent target cells where it binds to the haeme group in soluble guanylate cyclase and stimulates the production of cyclic GMP and the consequent relaxation of vascular smooth muscle cells. In addition to vascular relaxation, NO inhibits both platelet adhesion and aggregation (Radomski, *et al.*, 1987). The mechanism for cyclic GMP mediated inhibition of platelet activation is discussed above. Platelets also contain eNOS (Radomski, *et al.*, 1990) and are consequently a source as well as a target of NO. Radomski proposed that in platelets NO acts as an intracellular messenger inhibiting platelet aggregation.

NO generation from endothelial cells can be stimulated by physical stimuli, such as shear stress; as well as number of chemical agents, including acetylcholine bradykinin, serotonin and ADP (Shepherd & Katusic, 1991). Platelet aggregation and the consequent release of platelet derived serotonin, thromboxane and ADP may therefore be important in NO production from endothelial cells. Endothelium dependent relaxation induced by aggregating platelets in human mammary arteries can be inhibited by apyrase, an ADP scavenger and by LNMMA (Föstermann *et al.*, 1988).

Local infusion of LNMMA, the competitive inhibitor of NO production, into the human forearm causes a dose dependent reduction in resting forearm blood flow (Vallance, 1989), demonstrating that NO contributes to basal vasodilator tone by opposing vasoconstrictor forces. However, whether infusion of LNMMA alters platelet activity is not unclear. Systemic infusion of LNMMA has been shown to reduce bleeding time, but without affecting *ex vivo* platelet aggregation (Sigmon & Beierwaltes, 1995).



Impaired endothelium dependent relaxation has been reported in a number of conditions where abnormal platelet activity is also observed, such as diabetes (Poston & Taylor, 1995) and hypertension (Panza *et al.*, 1990) Indeed intravenous infusion of LNMMA has been shown to increase mean arterial pressure in healthy subjects (Haynes *et al.*, 1993) suggesting that a reduction in NO synthesis in hypertension might contribute to increased blood pressure. In atherosclerosis a decrease in endothelium dependent relaxation is likely to be linked to decreased NO production. This is consistent with the findings of Ludmer and colleagues, who showed that acetylcholine produced constriction rather than relaxation in atherosclerotic human arteries *in vitro* in the presence of the endothelium (Ludmer *et al.*, 1986). A reduction in NO production could contribute greatly to platelet deposition and the release of platelet derived growth factors known to be involved in the progression of atherosclerosis.

Angiotensin II is a potent vasoconstrictor octapeptide. Its substrate, angiotensinogen, is converted by proteolytic cleavage of the ten N-terminal amino acids by renin secreted from the kidney, to form the decapeptide angiotensin I. Angiotensin converting enzyme (ACE) cleaves two C-terminal amino acids from angiotensin I to form angiotensin II. Although high concentrations of ACE are found in the lung it is also present in the vascular endothelium, as is angiotensinogen and renin. Evidence for vascular production has come from isolated vessel studies where angiotensin II production was observed in the absence of circulating blood (Nakamaru *et al.*, 1986; Mizuno, 1991). Of the two angiotensin II receptors, it is the type I receptor which is thought to mediate the physiological actions of the peptide in the cardiovascular system. Angiotensin receptors were identified on human platelets in 1982 (Moore & Williams, 1982) and a functional interaction was first described in 1985 (Ding *et al.*, 1985b). Angiotensin II alone appears to have no direct effect on platelet aggregation. However, it modulates adrenaline-induced aggregation in a biphasic manner; at low

concentrations, it potentiates aggregation, and at higher concentrations it inhibits aggregation. It has also been shown to potentiate the effects of TxA<sub>2</sub>.

In vascular smooth muscle cells contraction induced by angiotensin II is mediated by an increase in intracellular calcium (Dostal *et al.*, 1990). A similar mechanism is observed in platelets (Haller *et al.*, 1989).

Although the physiological significance of the modulation of platelet aggregation by angiotensin II is not fully understood, angiotensin platelet binding and the effects of angiotensin on platelet calcium have been studied in a number of conditions where angiotensin sensitivity is thought to be altered, such as essential hypertension, pre-eclampsia and diabetes. Studies of the actions of angiotensin II on platelets in pre-eclampsia have demonstrated both an increase in intracellular calcium (Haller, *et al.*, 1989) and an increase in angiotensin binding (Baker *et al.*, 1991). In hypertension an increase in the angiotensin mediated calcium response was observed but receptor binding studies have not produced consistent results (Crabos *et al.*, 1993; Ding *et al.*, 1985a).

### Endothelin

In 1985 Hickey et al described a peptidergic endothelium derived constrictor, present in culture medium from bovine endothelial cells (Hickey *et al.*, 1985). Three years later this factor was isolated and sequenced from cultured porcine endothelial cells, and named ET (Yanagisawa *et al.*, 1988). A 21 amino acid peptide, ET is the most powerful vasoconstrictor known to date and induces a characteristically sustained effect.

Despite the apparently distinctive structure of ET, analysis of human genome revealed the existence of three ET peptides (Inoue *et al.*, 1989a; Inoue *et al.*, 1989b), named ET-1, -2 & -3. The ETs were also shown to have structural similarity to the snake venoms sarafotoxins (SRTX) isolated from the Israeli burrowing asp (Fig [1]), and unsurprisingly the SRTXs share some of the effects of the ETs. All ETs and SRTXs are made up of 21 amino acids, identical at ten positions including the four cysteines which contribute to the two disulphide bridges. ET-1 and -2 show the greatest homology differing only in amino acids 6 and 7.

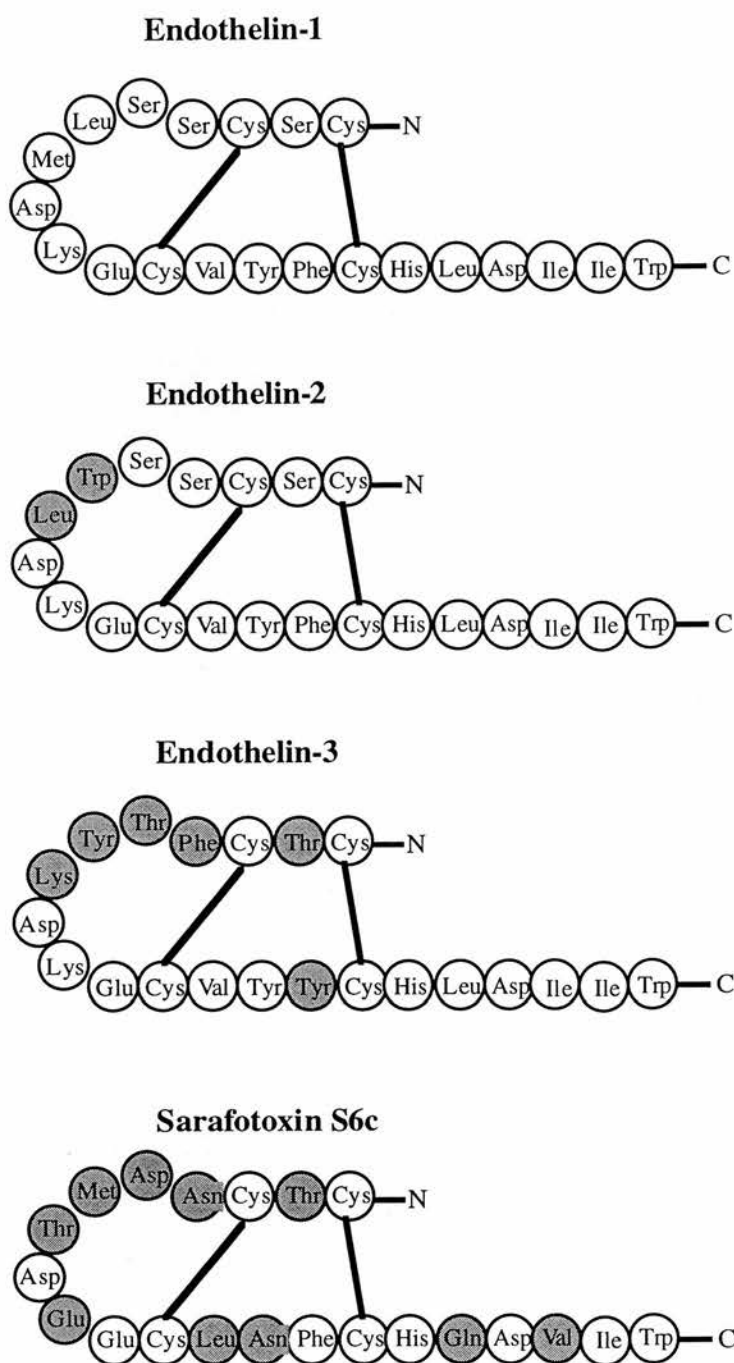


Figure 1.1. A diagrammatic representation of ET-1, 2 &3 and SRTX S6c

ET-1 is initially synthesised as a prepropeptide, as are ET-2 and ET-3, which undergoes a three step modification for the production of mature ET-1 (Inoue, *et al.*, 1989b). Prepro-ET-1 consists of 212 amino acids, which is cleaved at Arg52-Cys 53 and at Arg92-Ala93 by dibasic amino acid endopeptidase(s) to form the form, known as big ET-1. The 38 amino acid big ET-1 then undergoes further cleavage at Trp21-Val22 by an endothelin converting enzyme (ECE) to form the 21 amino acid ET-1. ECE is a phosphoramidon sensitive metalloprotease, as phosphoramidon has been shown to inhibit the conversion of exogenous big ET-1 to ET-1 (McMahon *et al.*, 1991) as well as causing a concomitant increase in big ET-1 and decrease in mature ET-1 secretion from cultured endothelial cells (Sawamura *et al.*, 1991). In 1994 Xu *et al* reported the cloning of ECE-1 (Xu *et al.*, 1994), an enzyme that readily converted big ET-1 to ET-1 at a neutral pH of 6.8, the activity of which was inhibited by phosphoramidon but not thiorphan distinguishing it from neutral endopeptidase-24.11. Intact cells transfected with the ECE-1 cDNA construct were able to secrete ET-1 and convert exogenous Big ET-1 to mature ET-1. They found that ECE-1 was expressed in a range of cell types, but expressed most abundantly in endothelial cells.

The following year, members of the same group reported the cloning of ECE-2 (Emoto & Yanagisawa, 1995). In an attempt to find an ECE that might be responsible for the production of mature ET-2 or ET-3 or explain ET-1 production in cells not containing ECE-1 they looked for structurally related isoenzymes of ECE-1, however like ECE-1, ECE-2 converts big-ET-1 to the mature peptide more efficiently than it does big ET-2 or -3. The main differences between the two isoforms are that ECE-2 has an acidic pH optimum (suggesting that it is localised in acidic intracellular compartments), is more sensitive to phosphoramidon and appears to be present in neuronal cells. Converting enzymes with preferential activity for big

ET-2 and -3 remain to be identified. Intracellular ECE activity may be inefficient or selectively regulated as big ETs are recognised to exist at measurable concentrations in human plasma (Miyauchi *et al.*, 1989). Blood cells can convert big ET-1 to ET-1 (Watanabe *et al.*, 1991) but this is not thought to be a major site of conversion.

Many stimuli induce ET production, on the basis of induction of ET-1 mRNA or elevated immunoreactive ET-1, (ir-ET-1), including adrenaline, angiotensin II, arginine vasopressin, transforming growth factor  $\beta$ 1, shear stress and hypoxia (Haynes & Webb, 1993a). In addition, aggregating platelets have been shown to induce ET-1 mRNA and increase ir-ET-1 without physical contact between the platelets and the endothelial cells (Ohlstein *et al.*, 1991). NO has been demonstrated to inhibit ET-1 production (Boulanger & Luscher, 1990).

Two ET receptors have been cloned and shown to be expressed in mammalian tissue. The initial cloning of the ET receptors occurred at approximately the same time. One receptor, named the ET<sub>A</sub>, was isolated from bovine lung cDNA (Arai *et al.*, 1990), and was demonstrated to have a high affinity for ET-1, the isoform selectivity being ET-1>ET-2>>ET-3. The other receptor, named ET<sub>B</sub>, was identified from the screening of a rat lung cDNA library (Sakurai *et al.*, 1990) and has equal affinity for all three ETs. SRTX S6c has been shown to be a selective agonist with a 30 000 fold greater selectivity for the ET<sub>B</sub> receptor than the ET<sub>A</sub> (Williams *et al.*, 1991). Both the ET<sub>A</sub> and the ET<sub>B</sub> receptors are similar, sharing the structure of G protein coupled receptor family, having seven transmembrane domains, separated by three extracellular and three intracellular loops, with a long extracellular N-terminal.

A third receptor, the ET<sub>C</sub> receptor, has been cloned from the frog *xenopus laevis* (Karne *et al.*, 1993). In the frog ET-3 was shown to activate this receptor with much greater potency than either ET-1 or ET-2 (ET-3>> ET-1=ET-2). Despite



pharmacological evidence for a receptor with the same rank order of potency in rabbit saphenous vein (Douglas *et al.*, 1995), the gene for such a receptor has yet to be identified in mammals. Variants within a receptor subtype may arise from alternative RNA splicing, such a variant has been identified for the ET<sub>B</sub> receptor. However, present evidence would suggest that this type of variant might result in alternative intracellular signalling rather than altered binding characteristics (Shyamala *et al.*, 1994).

The intracellular signal transduction mechanisms that mediate the actions of ET can, in the first instance be separated into pathways mediating short term changes and pathways mediating long term changes such as mitogenesis. The following section will address the mechanisms mediating short term changes.

#### ET intracellular signalling

Several mechanisms may be involved in ET-1 stimulation of cells, such as vascular smooth muscle cells. ET-1 stimulated increase in calcium may be by calcium influx as well as mobilisation of intracellular calcium, the latter most probably mediated by the PI system. Stimulation of phospholipase A<sub>2</sub>, phospholipase D and protein kinase C have also been suggested as intracellular pathways.

Much work has focused on direct activation of ET-1 stimulated calcium entry in an attempt to define the precise channel involved. Conflicting results on the involvement of L type calcium channels appear to depend on the source of the cells or tissue being examined. However, investigation into the ability of ET-1 to bind directly to, and activate, these channels found that ET-1 does not compete with L-type calcium channel ligand binding (Gu *et al.*, 1989). It remains likely that, at least

in part, ET-1 stimulated calcium entry might be subsequent to release of calcium from intracellular stores.

There is significant evidence for both ET<sub>A</sub> and ET<sub>B</sub> mediated stimulation of PI turnover in both cardiovascular tissues (Hilal-Dandan *et al.*, 1994; Ohlstein *et al.*, 1989) and transfected cells (Aramori & Nakanishi, 1992). As described above for platelets, activation of phospholipase C is the main mechanism for stimulation of PI turnover. Consequently, IP<sub>3</sub> mediated release of calcium could in turn mediate calcium entry. The other messenger generated by PI hydrolysis is DAG, and not surprisingly ET-1 stimulation of vascular smooth muscle cells is associated with a dose dependent increase in DAG (Griendling *et al.*, 1989).

Protein kinase C can be activated by DAG. ET-1 induced protein kinase C translocation has been reported in vascular smooth muscle cells (Lee *et al.*, 1989) and protein kinase C inhibition inhibited ET-1 induced contraction in rabbit aorta (Ohlstein, *et al.*, 1989). However, in the last study, like others, an increase in PI turnover was observed, and therefore, although it is clear that protein kinase C plays a role in ET induced effects it is not clear whether this kinase is stimulated directly or only subsequent to phospholipase C. Protein kinase C mediates the effects of the Na<sup>+</sup>-H<sup>+</sup> antiporter in many cell types, regulating intracellular pH. ET induced alkalisation was investigated in human resistance vessels (Richards *et al.*, 1989). Up to 70 % of contraction induced by ET-1 in these vessels appeared to dependent on Na<sup>+</sup>-H<sup>+</sup>; in vascular smooth muscle cells ET-1 stimulation of Na<sup>+</sup>-H<sup>+</sup> exchange was blocked by protein kinase C inhibition (Lonchampt *et al.*, 1991). This further supports the role of protein kinase C in the effects of ET and suggests that these effects are partly mediated by the regulation of intracellular pH.



The greatest differences in the intracellular mechanisms between ET<sub>A</sub> and ET<sub>B</sub> mediated responses appear to arise in the study of the cyclic nucleotide second messengers, cyclic AMP and cyclic GMP. Significant differences also seem to exist between different cell types. In transfected cells expressing ET<sub>A</sub> or ET<sub>B</sub> receptors, the ET<sub>A</sub> receptor was linked to a stimulation of adenylate cyclase and a consequent increase in cyclic AMP, whereas the ET<sub>B</sub> receptor mediated an inhibition of forskolin stimulated cyclic AMP (Aramori & Nakanishi, 1992). Care must be taken in the interpretation of studies involving transfected cells, as transfection with receptor DNA may not necessarily result in the same signal transduction as cells naturally expressing these receptors. Nevertheless, in alveolar epithelium cells the ET<sub>A</sub> receptor stimulation was associated with prostaglandin synthesis and cyclic AMP accumulation, and a significant increase in cyclic AMP was still observed in the presence of indomethacin (Markewitz *et al.*, 1995). However, in rabbit aorta ET<sub>A</sub> activation was not associated with any change in cyclic AMP (Ohlstein, *et al.*, 1989). In intact rat tail arteries ET-3 administration inhibited isoproterenol stimulated cyclic AMP (Yang *et al.*, 1991). The presence of the endothelium and the efficacy of ET-3 suggest that this may be ET<sub>B</sub> mediated. This is consistent with ET-3 inhibition of forskolin stimulated cyclic AMP in bovine endothelial cells (Eguchi *et al.*, 1993). ET-3 mediated activation is also associated with an increase in cyclic GMP, as seen in kidney epithelial cells (Ishii *et al.*, 1991).

In the human vasculature, early studies suggested that the ET<sub>A</sub> receptor, restricted to the smooth muscle cells, mediates contraction, and the ET<sub>B</sub> receptor, restricted to the endothelium, mediates relaxation. However, the recognition that ET<sub>B</sub> receptors are also present on smooth muscle cells (Davenport *et al.*, 1995), and can mediate contraction (Clozel *et al.*, 1992) forced a revision of this perspective. The endothelial ET<sub>B</sub> receptor is sometimes distinguished from the muscular ET<sub>B</sub> receptor by referring to them as ET<sub>B1</sub> and ET<sub>B2</sub> respectively.

A number of antagonists have been developed that distinguish between the ET<sub>A</sub> and the ET<sub>B</sub> receptors, as well as some that are thought to distinguish between the ET<sub>B1</sub> and ET<sub>B2</sub> receptors (Ferro & Webb, 1996). A commonly used ET<sub>A</sub> antagonist is BQ-123; potent and highly selective, BQ-123 antagonises ET-1 binding and ET-1 induced contraction at the ET<sub>A</sub> receptor (Ihara *et al.*, 1992). However, residual contraction in the presence of BQ-123 provided early evidence for ET<sub>B</sub> mediated contraction of rat aortic smooth muscle. An extensively used ET<sub>B</sub> receptor antagonist, BQ-788 has been shown not to affect ET<sub>A</sub> mediated pressor responses (Ishikawa *et al.*, 1994) but to antagonise both ET<sub>B1</sub> and ET<sub>B2</sub> mediated responses (Karaki *et al.*, 1994). The partial antagonism exhibited by certain ET<sub>B</sub> antagonists helped characterise ET<sub>B1</sub> and ET<sub>B2</sub> receptors. The antagonist PD 142893 is considered able to distinguish between the two putative subtypes (Douglas, *et al.*, 1995).

#### Endothelin in health and disease

Intravenous administration of ET-1 causes a transient vasodilation followed by a profound and long lasting increase in blood pressure (Yanagisawa, *et al.*, 1988). The initial vasodilator response was thought to be due to stimulated release of ET-1 derived NO or prostacyclin. However, cyclooxygenase blockade with indomethacin did not significantly alter the transient dilatation (Rubanyi & Polokoff, 1994) suggesting that it does not involve prostacyclin synthesis. Inhibition of NO synthesis has been shown to attenuate this response (Gardiner *et al.*, 1990). Consequently, NO release appears to be involved. In addition to direct vasoconstrictor effects at low concentrations, ET-1 also potentiates the effects of other constrictor agents such as norepinephrine and serotonin (Yang *et al.*, 1990; Nakayama, 1991).



The role of ET in disease is very much an open field. Increased circulating concentrations of ir-ET-1 have been reported in a number of pathophysiological conditions. Indeed, increased ET mediated responses have also been reported, yet until ET receptor antagonism, or ECE inhibition result in beneficial effects in man in a particular disease it remains speculation whether ET-1 has a causative role. The advent of orally active ET antagonists should help greatly in this respect. The evidence for a role of ET-1 in a few vascular conditions is discussed below.

Yanagisawa postulated that disturbances in the control of ET production could contribute to the pathogenesis of hypertension (Yanagisawa, *et al.*, 1988). However, plasma concentrations are not raised in patients with essential hypertension compared to age matched controls (Davenport *et al.*, 1990). An increased sensitivity to exogenous ET-1 has been shown in the dorsal hand vein of untreated hypertensive patients and if a similar increased sensitivity were to exist in other tissue it remains possible that ET may be involved in the pathophysiology of this condition.

The powerful vasoconstrictor properties of ET-1 and its role as a locally acting hormone suggest a possible role in vasospastic or ischaemic conditions. Indeed, exogenous ET-1 causes coronary vasoconstriction leading to myocardial ischaemia in a canine model (Kurihara *et al.*, 1989). Plasma levels of ir-ET-1 were reported to be significantly raised (Lam *et al.*, 1991) and suggested to be an early indicator of acute myocardial infarction in man (Stewart *et al.*, 1991). The number of cardiac binding sites for iodinated ET-1 are also reported to be raised after ischaemia (Liu *et al.*, 1990). Both the extent of infarction (Yasuda *et al.*, 1990) and the prognosis, post infarction, (Omland *et al.*, 1994) correlate with ET levels indicating that plasma ET-1 levels may be associated with the severity of myocardial infarction. But, perhaps, the most significant evidence for a direct role of ET-1 is that infusion of BQ-123

reduces the extent of experimentally induced myocardial infarction by up to 40 % in animal studies (Grover *et al.*, 1993).

Following subarachnoid haemorrhage (SAH) cerebral vasospasm develops. ET-1 has been implicated in the development of this potentially fatal secondary event. Both plasma (Masaoka *et al.*, 1989) and cerebrospinal fluid levels (Seiffert *et al.*, 1995) of ET-1 are raised in patients with SAH as they are in a canine models of the disease (Yamaura *et al.*, 1992). However, ECE inhibition, using phosphoramidon, reduced the vasospasm along with lowering cerebrospinal fluid ET-1 levels in the canine model (Matsumura *et al.*, 1991). Administration of BQ-123 has also been shown to reduce SAH induced vasospasm in dogs (Clozel *et al.*, 1993). The beneficial effects of inhibition of ET-1 production as well as ET receptor antagonism strongly supports a role for ET-1 in the development of post SAH vasospasm.

#### Endothelin and platelets

The discovery of an endothelium derived constrictor raised the question of whether ET-1 might also regulate platelet activity. It was already known that endothelium derived agents such as prostacyclin and EDRF (NO) inhibited platelet activity. However, at sites of haemorrhage, the balance is required to change in favour of platelet activation. ET-1 may play an important role in haemostasis at the site of haemorrhage, its release being stimulated with factors known to play a role in coagulation such as thrombin and adrenaline (Yanagisawa, *et al.*, 1988) and the consequent vasoconstriction limiting blood flow near a site of damage. In conditions such as hypertension and vasospasm, where ET-1 may have a pathophysiological role, platelet activity is increased and may contribute to the condition. Consequently, the possibility of ETs modifying platelet activity was investigated.



Early investigations failed to reveal any effect of ET-1 (Patel *et al.*, 1989) or ET-3 (Lidbury *et al.*, 1989) alone or in combination with other platelet aggregating agents on platelet activation *in vitro*. In both studies three aggregating agents were used; in the former ADP, thrombin and U44069, and in the latter ADP collagen and arachidonic acid. However, *ex vivo* studies, examining platelet aggregation after administration of systemic doses of ET-1 to dogs (Hermán *et al.*, 1989) and rabbits (Thiemermann *et al.*, 1989), showed an inhibition of platelet aggregation. In the canine study the inhibition of platelet aggregation coincided with an increase in plasma levels of 6-keto PGF<sub>1α</sub>, a metabolite of prostacyclin, and both the inhibition of platelet aggregation and the rise in 6-keto PGF<sub>1α</sub> were attenuated by pre-treatment with acetylsalicylic acid, a cyclooxygenase inhibitor. Hence, the inhibitory action of ET-1 on platelet aggregation was attributed to ET-1 stimulated prostacyclin release. Similarly, in the study reported by Thiemermann, pre-treatment with the cyclooxygenase inhibitor indomethacin markedly attenuated the ET-1 mediated inhibition of platelet aggregation. Interestingly, in this study a residual indomethacin insensitive ET-1 inhibition of platelet aggregation was observed up to 60 min after ET-1 administration.

The following year three further studies were published investigating the effect of ET-1 on platelet aggregation *in vitro*. Edlund & Wennmalm reported that ET-1 did not induce platelet aggregation in human plasma for up to 5 min after the addition of the peptide, nor did it alter aggregation induced by thrombin or ADP, observed for 30 sec after the addition of the aggregating agents (Edlund & Wennmalm, 1990). The inability of ET-1 to induce platelet aggregation within 5 min of addition to human plasma was confirmed by Ohlstein *et al.* (Ohlstein *et al.*, 1990). In this study ET-1 failed to affect platelet aggregation to varying doses of ADP and collagen or single doses of arachidonic acid or adrenaline selected to elicit half maximal responses. However, ET-1 did significantly potentiate aggregation to 'low'

concentrations of ADP (0.625 & 1.25  $\mu$ M) in rabbit and canine platelets. The third study published in 1990 reported a significant potentiation of adrenaline induced platelet aggregation *in vitro* in human platelets (Matsumoto *et al.*, 1990). In this study ET-1 dose dependently potentiated aggregation to 0.08 & 0.2  $\mu$ g/ml adrenaline 10 min after the addition of the aggregating agent. Interestingly, potentiation of aggregation was not seen in all subjects in this study. Unfortunately, there was no data on the subjects that might have helped identify the responders.

## Aims

At the outset of this work, the published outcome of studies investigating the actions of ET-1 on platelets provided intriguing and inconclusive results. Although the evidence suggested that ET-1 did not itself elicit platelet aggregation when observed over a 10 min period, it might potentiate the effect of 'weak' platelet aggregating agents *in vitro*. In addition, ET-1 appeared to inhibit *ex vivo* platelet aggregation in rabbits by an unidentified mechanism up to 60 min after ET-1 administration.

It was, therefore, the aim of this work: to establish

1. whether ET-1 acts directly on human platelets;
2. to characterise the receptor(s) involved; and
3. to investigate the actions of ET-1 on platelets in diseases where ET-1 is thought to have a pathophysiological role.

## Hypotheses

From the available literature it was hypothesised that ET-1 can both potentiate and inhibit platelet aggregation by acting on specific platelet surface receptors and that the pro-aggregatory and inhibitory effects are mediated by different ET receptors. In Furthermore, it was hypothesised that in essential hypertension the action of ET-1 on platelets may relate to the pro-aggregatory state of platelets in this condition.

The studies arising from these hypotheses were:

to investigate: whether or not ET-1 causes aggregation of human platelets *in vitro*, by the use of Born light transmittance aggregometry (Born, 1962) and whether ET-1 modulates the actions of established platelet aggregating agents;

to characterise the ET receptor(s) which mediated the putative effects of ET-1 on human platelets;

to explore the role of second messenger cyclic nucleotides in the modulation of platelet activity by ET receptor agonists;

to establish the existence of cell surface receptors by ligand binding studies and analysis of residual platelet mRNA;

to investigate whether human platelets exposed to ET-1 *in vivo* exhibit similar responses when analysed *ex vivo* to responses obtained from *in vitro* studies, and to examine the role of cyclooxygenase products on these responses;

and to investigate the effects of blood pressure and the presence of essential hypertension on the effects of ET-1 on platelet aggregation and cyclic nucleotide levels.



## **CHAPTER 2.**

### **MATERIALS & METHODS**

## METHODS

### Platelet Aggregation

Subjects participating in aggregation studies were healthy males between the ages of 18 and 50 years. All were required to abstain from caffeine and nicotine for a minimum of 2 hr prior to venesection, and aspirin or non-steroidal anti-inflammatory drugs for the ten days prior to venesection. On the day of venesection subjects were rested supine in a quiet room for 20 to 30 min and blood was taken from a vein in the cubital fossa via a 19 gauge needle, without tourniquet and with minimum stasis, into 10 ml plastic tubes containing 1 ml acid citrate dextrose; 9:1, final concentration: citric acid 8 mg ml<sup>-1</sup>, sodium citrate 22 mg ml<sup>-1</sup>, glucose 20 mg ml<sup>-1</sup>. Blood was promptly centrifuged at room temperature for 10 min at 120 g in a Gallenkamp Bench-top centrifuge to obtain platelet rich plasma (PRP). PRP was pooled and 890 µl aliquots were pipetted into 2 ml plastic tubes, gassed with 95% O<sub>2</sub>:5% CO<sub>2</sub> capped and stored at room temperature until required. The remaining blood was further centrifuged at 3000 g for 20 min at room temperature to obtain platelet poor plasma (PPP). PPP was pooled and a single aliquot of 890 µl was mixed with 110 µl normal saline.

Aggregation studies were carried out 1 hour after venesection to allow responses to stabilise (Siess *et al.*, 1981). Platelet aggregation was measured by light transmittance according to the method described by Born (Born, 1962), based on a decrease in light transmittance through the sample as platelets come out of suspension to form aggregates (Fig 2.1). Samples are maintained at 37°C, stirred at 1200 rpm and allowed to equilibrate for 3 min prior to the addition of any agents. Light transmittance through the PRP sample, where all samples remain in

suspension, was taken as 0% aggregation and through the PPP sample, where there are no platelets in suspension, as the theoretical maximum aggregation of 100%. The maximal increase in light transmittance after the addition of the drug is expressed as a percentage aggregation.

Aggregation studies were performed in a Malin 6 channel aggregometer interfaced to a Macintosh LC computer by a MacLab analogue digital converter. The computer application used to record aggregation studies was Chart/8 V3.1.1. and the spreadsheet application used to calculate percent aggregation was Microsoft Excel version 3. Using this apparatus the maximum aggregation for each study was calculated by selecting three areas from the Chart document, where light transmittance was expressed in mV and transferring them to an Excel spread sheet. The three areas chosen were: a 10-15 sec period prior to aggregation to establish the base line 0% aggregation; mean light transmittance through PPP for 10-15 sec, to record the theoretical maximum; and the maximum light transmittance during the course of the study.

To establish how long platelets remained viable and produced reproducible aggregatory responses a validation study was carried out.

Six male subjects participated in this study under the conditions described above. Aggregatory responses were recorded to vehicle alone and three concentrations of adrenaline; low ( $0.01 \mu\text{g ml}^{-1}$ ), medium ( $0.1 \mu\text{g ml}^{-1}$ ), and high ( $1 \mu\text{g ml}^{-1}$ ). Platelet aggregation was measured at 5 time points; 60, 90, 120, 150 and 180 min after venesection. Aggregatory responses at each time point were recorded in parallel in separate aliquots of PRP.

No significant changes in the aggregatory responses were detected by repeated measure ANOVA up to 150 min after venesection. Aggregation to medium and high doses of adrenaline were significantly depressed at 180 min after venesection (Fig 2.2).

## **Cyclic Nucleotide Measurements**

### *Plasma Extraction*

Cyclic AMP and cyclic GMP were extracted from plasma by the following method. Ethanol was added to plasma, at a ratio of 2:1, the sample was immediately mixed using a Fisons Whirlimixer for approximately 10 sec, and then incubated at room temperature for 15 min. Samples were subsequently centrifuged at 1500 g for 15 min at 4°C, the supernatant decanted into glass tubes, and the protein pellet discarded. The samples were dried down under air at 55°C and reconstituted in assay buffer, and frozen at -20°C until assayed.

The efficiency of the cyclic nucleotide extraction protocol was assessed by 'spiking' plasma with <sup>125</sup>I-iodinated cyclic nucleotide. Ten µl of <sup>125</sup>I labelled cyclic AMP or cyclic GMP, with an approximate activity of 10 000 cpm, was added to 1 ml aliquots of plasma, the plasma was subjected to the extraction protocol described above and the radioactivity in the sample was measured after reconstitution in assay buffer.

The recovery of <sup>125</sup>I-cyclic AMP and <sup>125</sup>I-cyclic GMP did not differ significantly. The mean percentage recovery of <sup>125</sup>I-iodinated cyclic nucleotides was  $77.4 \pm 7.8\%$ , n= 6.

Cyclic nucleotides were measured by an in-house radioimmunoassay (RIA) developed according to the method described by Brooker (Brooker *et al.*, 1979).

#### <sup>125</sup>I-Iodination of cyclic nucleotides

<sup>125</sup>I-Iodination procedures were performed in controlled ventilated fume hoods. Succinyl cyclic AMP tyrosine methyl esters (ScAMP-TME) and succinyl cyclic GMP tyrosine methyl esters (ScGMP-TME), were prepared in assay buffer at a concentration of 60 µM. Na<sup>125</sup>I, 0.5 mCi was mixed with 10 µl 0.5 M potassium phosphate buffer pH 7.0, by repeated pipetting; 20 µl of ScAMP-TME or ScGMP-TME was added and mixed in the same fashion. The reaction was initiated by the addition of 10 µl chloramine T at a concentration of 1 mg ml<sup>-1</sup> in 0.5 M potassium phosphate buffer, pH 7.0. After 60 sec the reaction was stopped by the addition of 50 µl sodium metabisulphate, 12 mg ml<sup>-1</sup> in 0.5 M potassium phosphate buffer pH 7.0. Iodination of ScAMP-TME and ScGMP-TME were performed in parallel.

The cyclic nucleotide derivatives were purified by descending paper chromatography. The reaction mixture was spotted approximately 4 cm from one end of a Whatman 31ET paper strip, 2 cm by 40 cm. The end of the strip was submerged in the mobile phase, consisting of butan-1-ol:glacial acetic acid:distilled water (12:3:5), and held in position by a glass rod. After chromatography, of duration of 3-4 hr, the strip was dried, wrapped in cling film and autoradiographed. The radioactive areas on the strips, identified by superimposing the autoradiograph over the strip, were cut out and eluted in assay buffer:propan-1-ol (1:1). This protocol results in three radioactive 'peaks'; with R<sub>f</sub> values of approximately 0.4, 0.6 and 0.9-1.0, corresponding to free iodine, <sup>125</sup>I-TME cyclic AMP and the solvent

front respectively for cyclic AMP and Rf values of 0.4, 0.5 and 0.9-1.0 corresponding to free iodine,  $^{125}\text{I}$ -TME cGMP and the solvent front for cyclic GMP.

## Antibodies

Specific antibodies to cyclic AMP and cyclic GMP were raised in rabbits by Dr B C Williams and Dr I F Gow using an homologous bridge strategy (Brooker, *et al.*, 1979). The antibody for cyclic AMP was identified as R1B6 and for cyclic GMP as ED2-3.

For each new batch of  $^{125}\text{I}$ -iodinated cyclic nucleotide prepared, antibody dilution curves were performed to establish the antibody concentrations required for an approximate 50% binding of  $^{125}\text{I}$ -iodinated ligand in the absence of cold ligand.

## Antibody dilution curves

Antibody dilution curves were performed for  $^{125}\text{I}$ -iodinated cyclic AMP and cyclic GMP. The respective antibodies were prepared, by sequential double dilution in assay buffer, to initial concentrations of 1:1 000 to 1:128 000.  $^{125}\text{I}$ -iodinated ligand (150  $\mu\text{l} \equiv 4 - 6\ 000\ \text{cpm}$ ) were incubated with 100  $\mu\text{l}$  of antibody of the varying dilutions and 50  $\mu\text{l}$  of assay buffer, acetylated as described below. Preparation and incubation of samples were performed in the same manner as for the RIAs described below. Examples of typical antibody dilution curves are given in Fig 2.3.



## Assay Protocol

Standards for both cyclic AMP and cyclic GMP assays were diluted from stock solutions of 32  $\mu\text{M}$  in assay buffer (0.05 M sodium acetate buffer, pH 4.8) at concentrations of 0, 0.06, 0.12, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 nM.

## Acetylation

All samples and standards were acetylated in glass tubes prior to assay. Acetylating reagents, triethylamine and acetic anhydride, were premixed in glass tubes in a ratio 2:1 v/v. The premix of acetylating agents (15  $\mu\text{l}$ ) was added to 500  $\mu\text{l}$  of sample or standard and immediately mixed for 5 sec.

$^{125}\text{I}$ -iodinated ligand (150  $\mu\text{l}$   $\equiv$  4-6 000 cpm), antibody (100  $\mu\text{l}$ ) at the predetermined dilution and acetylated standard or sample were mixed in 3 ml plastic tubes. Samples were incubated at 5-8°C for 18 hr. Separation of antibody-bound ligand from free ligand was performed by centrifugation. Activated charcoal (700  $\mu\text{l}$ ) was added to each tube prior to centrifugation at 1700 g for 30 min at 4°C. The supernatant was aspirated off and discarded. The radioactivity in the remaining pellet (the 'free' fraction) was measured. The sample concentrations of cyclic AMP or cyclic GMP were calculated from the respective standard curves (Fig 2.4). All assays were performed in duplicate, and additions of RIA agents and charcoal were performed 'on ice'. The cyclic AMP assay has an intra- and inter-assay coefficient of variation of 5.2% and 8.1% respectively for a sample with assay value of 1.0 nM (n=5). The cyclic GMP assay has an intra- and inter-assay coefficient of variation of 4.1% and 7.2% respectively for a sample with assay value of 1.0 nM (n=5).

### *Platelet cyclic nucleotide measurements*

Validation of platelet cyclic nucleotide measurement in response to agonists was performed with agents known to stimulate cyclic AMP and cyclic GMP, PGE<sub>1</sub> and sodium nitroprusside (SNP) respectively.

To distinguish between plasma and platelet concentrations of cyclic nucleotide, platelets were separated from plasma prior to extraction. After incubation with PGE<sub>1</sub> or SNP, PRP was transferred to 1.5 ml conical polypropylene tubes pre-chilled at 4°C and immediately centrifuged at 10 000 *g* for 2 min at 4°C. The plasma and the platelet pellet were transferred to separate 4 ml plastic tubes and mixed vigorously with 2 ml ethanol, and the extraction was carried out as described above. Extracts from both platelet pellets and plasma were assayed.

Initial time course studies were carried out to establish the required incubation time with agonists for optimal stimulation of cyclic nucleotide.

Plasma was prepared in the same manner as described for platelet aggregation. After PRP had been stirred for 3 min at 37 °C, PGE<sub>1</sub> (1 µM) or SNP (10 µM) was added. The reactions were stopped and extractions performed at 0.5, 1, 3, 5 and 10 min. Time course studies for each agonists were performed in parallel.

PGE<sub>1</sub> (1 µM) produced a significant increase in both platelet and plasma cyclic AMP with time ( $p < 0.05$ ), peaking at 5 min. However, no significant increase was seen in either platelet or plasma cyclic GMP levels. Platelet cyclic nucleotide responses to PGE<sub>1</sub> are depicted in Fig 2.5. SNP, 10µM, produced highly variable cyclic AMP changes with time, which were not statistically significant. SNP did produce time dependent increases in both plasma and platelet cyclic GMP ( $p < 0.05$ ),



with a maximum increase in plasma cyclic GMP at 5 min. Platelet cyclic nucleotide responses to SNP as a function of time are depicted in Fig 2.6.

In the second part of the validation study dose response studies were carried out with PGE<sub>1</sub> (0.1-10  $\mu$ M) and SNP (0.1-10  $\mu$ M).

The protocol for plasma preparation and cyclic nucleotide extraction were carried out as described above; based on the data from the time course study, incubation of the agonists in plasma was stopped after 5 min. Dose response studies for each of the agonists (0.1 -10  $\mu$ M) were performed in parallel.

A dose dependent increase in cyclic AMP in response to PGE<sub>1</sub> was observed in both plasma and platelet extracts ( $p<0.05$ ). PGE<sub>1</sub> also produced a significant increase in platelet cyclic GMP ( $p<0.05$ ), but no significant change was seen in plasma cyclic GMP. Platelet cyclic nucleotide responses to increasing concentrations of PGE<sub>1</sub> are depicted in Fig 2.7. SNP did not produce any significant change in platelet cyclic AMP levels but did appear to produce a slight but significant rise in plasma cyclic AMP ( $p=0.05$ ). The rise in plasma cyclic GMP was also slight but statistically significant whereas, SNP induced a considerable rise in platelet cyclic GMP ( $p<0.05$ ). Platelet cyclic nucleotide responses to increasing concentrations of SNP are depicted in Fig 2.8.

## **Receptor Binding Studies**

### *Rat cardiac membranes*

Cardiac membranes preparation and binding was performed by an adaptation of a previously described method (Waugh *et al.*, 1992). Ventricles from adult male

Sprague Dawley rats (250-300 g) were roughly minced manually and washed in 50 ml ice-cold saline (0.9%). The tissue fragments were homogenised in 20 mM NaHCO<sub>3</sub> buffer, 0.1 mM phenylmethanesulphonylfluoride (PMSF), using two 10 s bursts of a Ystral Gmb-H homogeniser operating at 8/10 of maximum speed. The homogenate was centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was further centrifuged at 20 000 *g* for 15 min and the pellet resuspended and homogenised in 40 ml, 50 mM Tris buffer, 0.1 mM PMSF pH 7.4. Centrifugation was repeated twice. On the final occasion the pellet was resuspended as above at a concentration of 2 mg ml<sup>-1</sup>.

Binding was performed in 12 x 75 mm glass tubes in duplicate for 60 min at 37°C. All assay constituents were prepared in assay buffer, 50 mM Tris buffer containing 0.1 mM PMSF pH 7.4. The assay consisted; of 'total tubes', incubated in the absence of cold ET-1, non-specific binding tubes incubated in the presence of 10<sup>-6</sup> M ET-1 and tubes containing cold ET-1, final concentration 10<sup>-12</sup> - 10<sup>-8</sup> M increasing in log order magnitude. Membranes and <sup>125</sup>I-ET were diluted in assay buffer to give a final concentration of 0.25 mg/tube and 0.5 nM respectively. Constituents of the assay are given in table 2.1.

The reaction was stopped by the addition of 3 ml ice-cold 50 mM Tris buffer pH 7.4. Bound and free <sup>125</sup>I ET-1 were immediately separated by rapid filtration across Whatman GF/B glass microfibre filters, pre-soaked in 50 mM Tris buffer. The tubes were rinsed with a further 3 ml of buffer, which was subsequently applied to the filters. The filters were washed 3 times with 10 ml additions of the above buffer. Radioactivity of the filters was assessed by counting for 3 min in a multiwell gamma counter.

Table 2.1.

Additions	Total (μl)	NSB (μl)	Drug (μl)
Buffer	350	300	300
Radioligand ( <sup>125</sup> I-ET-1)	50	50	50
NSB (ET-1 10 <sup>-6</sup> M)	-	50	-
Competing Drug	-	-	50
Membrane	100	100	100
Total Volume	500	500	500

#### *Human platelet membranes*

Platelet membranes were prepared as previously described (Cheung *et al.*, 1982). Blood was collected into 3.5% sodium citrate (w/v). PRP was prepared by centrifugation at 200 g for 10 min at room temperature. PRP was centrifuged at 20 000 g for 15 min at 4°C. The resulting pellet was resuspended in 5 mM Tris buffer, 5 mM EDTA, pH 7.4. The pellet was washed in the above buffer by resuspension, homogenisation, using two 10 s bursts of a homogeniser, and centrifugation. The pellet was resuspended in 50 mM Tris buffer, 0.5 mM EDTA, pH 7.4, incubated for 15 min at 37°C, then washed in the same manner as described above. The final pellet was resuspended in 50 mM Tris buffer, 0.5 mM EDTA and stored at - 80°C until used in the binding assay.

### Platelet yohimbine binding assay

Yohimbine binding studies were carried out as described previously (MacKinnon *et al.*, 1992). All agents were prepared in the assay buffer, 50 mM Tris buffer, 0.5 mM EDTA, pH 7.4. The assay, performed in duplicate, consisted of; 'total binding' tubes, non specific binding tubes, achieved by incubation in the presence of 10  $\mu$ M phentolamine, and tubes containing cold yohimbine ( $10^{-11}$  -  $10^{-5}$  M final concentration), increasing in half log order concentrations. Membranes and  $^3$ H-yohimbine were diluted to give final assay concentrations of 0.2 mg/tube of protein and 2 nM, respectively. Incubation was for 15 min at 37°C. Volumes of the constituents of the assay are given in table 2.2.

The reaction was stopped by the addition of 3 ml ice-cold 50 mM Tris buffer, 0.5 mM EDTA pH 7.4. Bound and free  $^3$ H yohimbine were immediately separated by rapid filtration across Whatman GF/B glass microfibre filters, pre-soaked in 50 mM Tris buffer. The reaction tubes were rinsed with a further 3 ml of buffer, which was subsequently applied to the filters. The filters were washed 3 times with 10 ml additions of the above buffer. Filters were subsequently placed in scintillation mini-vials, to which 4 ml of scintillation cocktail was added. Tritium was counted in the Packard Tri-carb 4000 scintillation counter, with a counting time of 5 min per sample.

### Platelet endothelin binding assay

Binding was performed in 12 x 75 mm glass tubes in duplicate for 60 min at 37°C. All assay constituents were prepared in assay buffer, 50 mM Tris buffer, 0.5 mM EDTA, 0.1 mM PMSF pH 7.4. The assay consisted; of 'total tubes', 'non specific

binding tubes' achieved by incubation in the presence of  $10^{-6}$  M ET-1 and tubes containing cold ET-1 (final concentration  $10^{-12}$  -  $10^{-8}$  M) increasing in log order magnitude. Membranes and  $^{125}\text{I}$ -ET-1 were diluted to give final assay concentrations of 0.2 mg/tube of protein and 0.5 nM, respectively. Constituents of the assay are given in table 2.2.

The reaction was stopped by the addition of 3 ml ice-cold 50 mM, Tris 0.5 mM EDTA buffer pH 7.4, bound and free  $^{125}\text{I}$ -ET-1 were immediately separated by rapid filtration across Whatman GF/B glass microfibre filters, pre-soaked in 50 mM Tris buffer. The tubes were rinsed with a further 3 ml of buffer, which was subsequently applied to the filters. The filters were washed 3 times with 10 ml additions of the above buffer. Radioactivity of the filters was assessed by counting for 3 min in a multiwell gamma counter.

Table 2.2

Additions	Total ( $\mu\text{l}$ )	NSB ( $\mu\text{l}$ )	Drug ( $\mu\text{l}$ )
Buffer	100	-	-
Radioligand ( $^3\text{H}$ yohimbine/ $^{125}\text{I}$ -ET-1)	100	100	100
NSB (phentolamine $10^{-5}\text{M}$ /ET-1 $10^{-6}\text{M}$ )	-	100	-
Competing Drug	-	-	100
Membrane	300	300	300
Total Volume	500	500	500

## **Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

Platelets were prepared by two separate methods. The first protocol was an adaptation of the method described by Shibata (Shibata *et al.*, 1994). Fifty five ml of venous blood was mixed with 5 ml of 3.2% sodium citrate. PRP was prepared by centrifugation at 100 *g* for 10 min at 22°C. PRP was diluted 20 fold in Medium 199 buffer containing 5 mM EDTA and 0.2% bovine serum albumin, and centrifuged at 1000 *g* for 10 min at 4°C. The pellet was resuspended in Medium 199 and both supernatant and resuspended pellet were centrifuged at 1000 *g* for 20 min at 4°C both subsequent pellets were snap frozen in liquid nitrogen, and identified as platelet rich pellet and platelet poor pellet.

Analysis of platelet pellets prepared in this manner was performed by the Department of Clinical Haematology at the Western General Hospital, Edinburgh. The platelet rich pellet resulted in approximately 10 fold greater harvest of platelets compared to the platelet poor pellet, and although both preparations had a mean contamination of less than 1:1000 white blood cells, white blood cells were only detected in 2 of 6 platelet poor pellet preparations.

The second protocol for the preparation of platelets involved purification of platelets on discontinuous gradients of percoll as described by Nubile (Nubile *et al.*, 1989). Percoll was prepared in saline at 4 densities (1.040, 1.060, 1.070 and 1.080). PRP was prepared as described above. PRP was mixed with apyrase (7.5 U/ml) and centrifuged for a further 20 min at 500 *g*. The resulting pellet was reconstituted in 3 ml of saline and layered gently onto the percoll. The percoll was centrifuged for 30 min at 400 *g* at 4°C. The relative density of the bands of percoll was verified by simultaneous centrifugation of percoll containing density marker beads. The band of percoll containing the platelets, 1.060 density, was removed and centrifuged at 1000



g for 20 min the subsequent pellet was snap frozen in liquid nitrogen and stored at -80°C.

The purity of the platelet pellet was verified by the Department of Clinical Haematology at the Western General Hospital, Edinburgh. No whole cells other than platelets were detected in three samples prepared from separate 20 ml aliquots of PRP.

#### *RNA extraction*

RNA extraction was performed by an adaptation of the method previously described by Chomczynski (Chomczynski & Sacchi, 1987). After defrosting, pellets were mixed with 1 ml of solution D (given in appendix 2.2). The pellet was 'homogenised' by being repeatedly drawn through a 24 gauge needle. The homogenate was then transferred to a 14 ml polypropylene tube. Sodium acetate, 2M, pH 4, was added and mixed by gentle inversion. One ml of water saturated phenol was added and mixed in the same manner, 0.2 ml of chloroform:isoamyl alcohol mixture (49:1) was then added and vigorously mixed for 10 sec. The final suspension was cooled on ice for 15 min. Samples were centrifuged for 20 min at 10 000 g at 4 °C. The aqueous phase was transferred to a fresh tube and mixed with 4 µl of glycogen (20 µg/µl) and 1 ml of isopropanol, and incubated at -20°C for 60 min. After a further centrifugation at 10 000 g for 20 min at 4°C the supernatant was carefully removed, the pellet was resuspended in 0.3 ml of solution D and transferred to a 1.5 ml eppendorf tube. The centrifuged tube was rinsed with 0.1 ml of solution D, which was added to the eppendorf. Precipitation of RNA was achieved by the addition of 0.4 ml of isopropanol and incubation for 60 min at -20°C. The eppendorf was centrifuged at 4°C for 20 min and the supernatant carefully removed. The pellet was gently lifted from the bottom of the eppendorf by the addition of 500 µl of 70% ethanol, and the

ethanol was subsequently decanted. The pellet was dissolved in 1 mM EDTA pH 8 solution treated with diethyl pyrocarbonate (DEPC), and stored at -20°C.

### *cDNA synthesis*

Before reverse transcription, total RNA was measured by optical density. The total recovered RNA, in a volume of 13 µl, was mixed with 1.5 nmol of random nucleotide hexamers, in 1.5 µl and heated to 70 °C for 5 min, then placed on ice. The RNA was added to a mixture of; 17.5 U RNase inhibitor, 35 U µl<sup>-1</sup>, 5 µl of RT buffer (constituents given in appendix 2), 2.5 µl of dithiothreitol (DTT), final concentration 10 mM, 1.2 µl of dinucleotide triphosphates (dNTP) mixture 0.6 mM of each nucleotide and 1µl of Moloney Murine Leukaemia Virus reverse transcriptase (MMLV), 200 U ml<sup>-1</sup>. The total mixture was incubated at 37°C for 60 min followed by inactivation of the reverse transcriptase by incubation at 95°C for 5 min, centrifuged briefly and placed on ice. An aliquot of 4 µl of DNA was removed for PCR and the remainder frozen at -20°C.

### *PCR*

The cDNA synthesised by reverse transcription was used for a template for PCR amplification with selective oligo nucleotide primers for ET<sub>A</sub> and ET<sub>B</sub> receptors (Elshourbargy *et al.*, 1993). The reaction mixture was prepared and 40 µl of paraffin was gently layered on top. The mixture was heated for a 'hot start' before the primers were added through the paraffin. Details for the conditions for PCR are given in table 3.1. The reaction was terminated by 10 min incubation at 72°C and stored at 4°C.

PCR products were purified and identified by gel electrophoresis, in a 1.2% agarose gel, made up in TBE buffer (constituents given in appendix 2).



Table 2.3

	ET <sub>A</sub>	ET <sub>B</sub>
Primers		
Forward	5` (AATCTG)CAGGGC	5` (AACGAATTCA)GC
Reverse	ATCCTTTTGGCTGGC AC 5` (ACAGGGATCC)GG CAAGACTGGCTATCA GCG	AAACCGCAGAGATA ATGAC 5` (TAAGGATC)CACG AACACAAGGCAGGA CAC
Constituents of mix.	31.7 µl DEP H <sub>2</sub> O, 0.8 µl dNTP, 12.5 mM, 4 µl MgCl <sub>2</sub> , 25 mM, 5 µl Taq buffer, 0.8 µl Taq, 5 Uµl <sup>-1</sup> , 4 µl cDNA	33.5 µl DEP H <sub>2</sub> O, 0.8 µl dNTP, 12.5 mM, 4 µl MgCl <sub>2</sub> , 25 mM, 5 µl Taq buffer, 0.8 µl Taq, 5 Uµl <sup>-1</sup> , 4 µl cDNA
Primers	250 nM in 1.9 µl 0.9 µl of forward primer 1.0 µl of reverse primer	250 nM in 3.7 µl 2.0 µl of forward primer 1.7 µl of reverse primer
Conditions for		
Hot Start	92 °C for 5 min 66 °C for 3 min 29 cycles of	94 °C for 5 min 64 °C for 3 min 29 cycles of
Extension	74 °C for 1 min	72 °C for 1 min
Denaturing	92 °C for 1 min	92 °C for 1 min
Annealing	66 °C for 1 min	64 °C for 1 min

**Forearm Blood Flow**

Forearm blood flow was measured according to the method previously by Haynes (Haynes *et al.*, 1991). The left brachial artery was cannulated under local anaesthesia with a 27 gauge steel needle. Saline (0.9%) and ET-1 (infusate concentration 5 nM) were infused at a rate of 1 ml min<sup>-1</sup> via a Welmed P 1000 syringe pump. Forearm

blood flow was measured in the infused and non-infused arm by venous occlusion plethysmography. The hands were excluded from circulation during each measurement period by inflation of a wrist cuff to 220 mm Hg. The last 5 individual flows in each 3 min measurement period were averaged and used for analysis. The percentage change in blood flow was calculated in accordance with the method described by Webb (Webb, 1995), as percent change in the ratio of blood flow (infused arm / control arm) compared with the ratio at the last baseline recording.

### **Endothelin Radioimmunoassay.**

Plasma ET was measured using a commercially available radioimmunoassay kit. Venous blood was collected into 10 mM EDTA and centrifuged at 2000 g for 15 min. The plasma was transferred to a separate tube with care, to avoid platelet and white cell contamination, and stored at -20°C.

ET was extracted from 2 ml of plasma, acidified with 3 ml 4% acetic acid in distilled water, onto a C18 Sep-Pak column, pre-conditioned by sequential additions of 5 ml ethanol, 5 ml distilled water and 5 ml 4% acetic acid in distilled water. Sep Paks were washed by separate additions of 3 ml distilled water and 3 ml 25% ethanol, and allowed to elute under gravity. ET was eluted, under gravity, from the column by two rinses with 1 ml each of 4% acetic acid in 86% ethanol. The pooled eluant was dried down at 37°C under nitrogen, reconstituted in ET assay buffer and stored at -20°C.

The assay kit was a standard radioimmunoassay based on competition between cold ET-1 and  $^{125}\text{I}$ -ET-1 for binding to an anti-ET rabbit antibody. Precipitation of bound fraction was by incubation with an anti-rabbit IgG antibody. Provided standards were in the range 8 -192 pg ml<sup>-1</sup>. The Coefficient of Variation for intra-

and inter-assay variation were 4.5%, for a mean value of 14.7 pg ml<sup>-1</sup>, and 6.8%, for a mean value 10.9 pg ml<sup>-1</sup>, respectively. The assay has a 52% cross reactivity for ET-2, a 96% cross reactivity for ET-3 and a 7% cross reactivity for Big-ET. Cross reactivity for other endogenous peptides such as angiotensin II and vasopressin were < 0.1%.

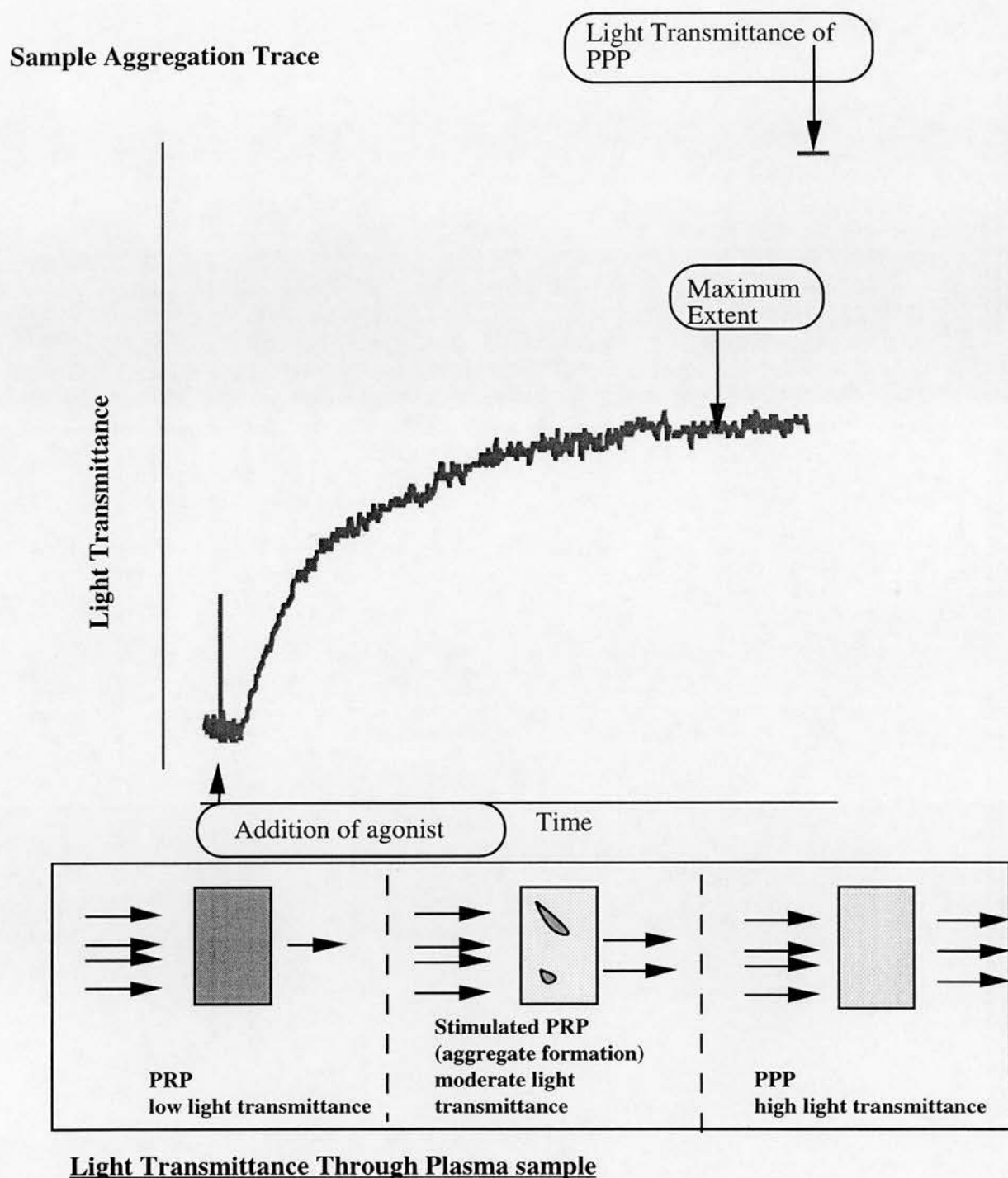


Figure 2.1. A sample aggregation trace with a representation of light transmittance through a plasma sample, illustrating increased light transmittance with aggregate formation.

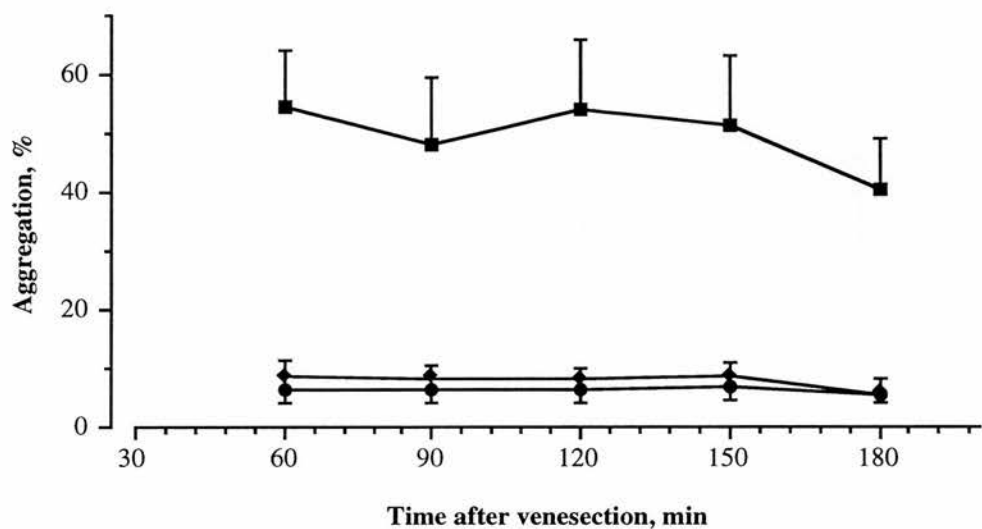


Figure 2.2 Reproducibility of aggregatory response with time. Low ● , medium ◆ and high ■ concentrations of adrenaline

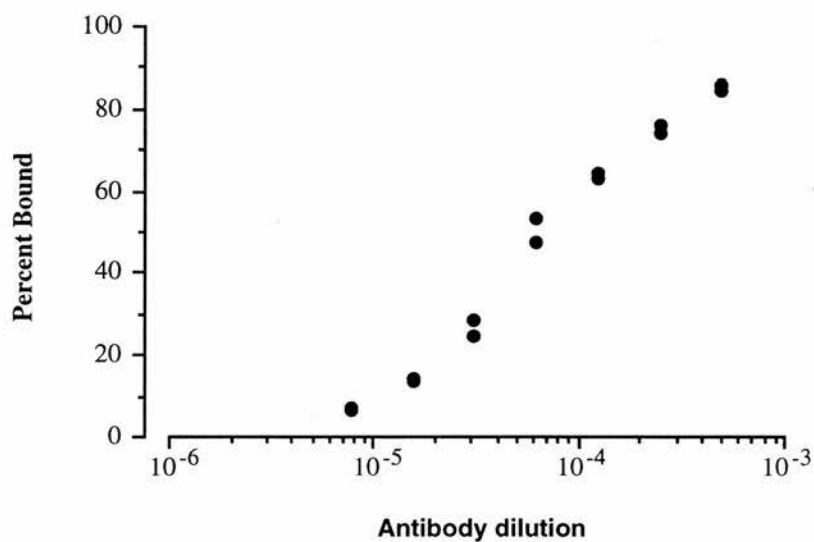


Figure 2.3 Antibody dilution curve for ED2 -3, cyclic GMP antibody, performed in duplicate, showing a 50% '0 binding' of 1:15 000 ( $6.7 \cdot 10^{-5}$ ).

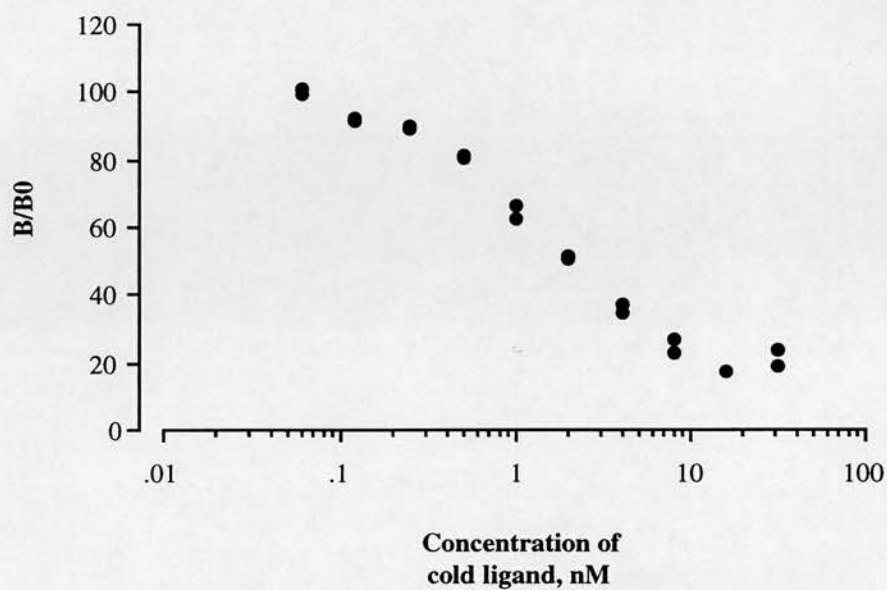


Figure 2.4a. cyclic AMP standard curve, performed in duplicate.

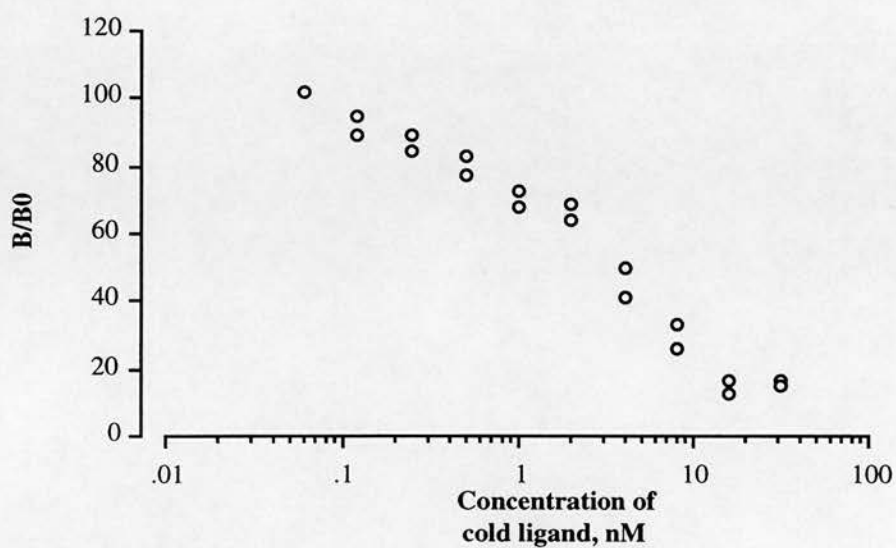


Figure 2.4b. cyclic GMP standard curve, performed in duplicate.

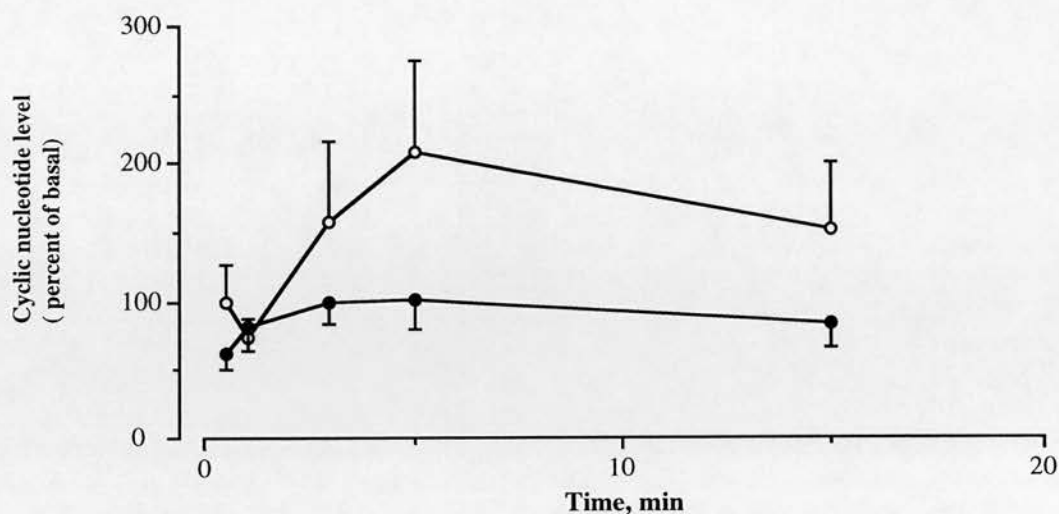


Figure 2.5. Cyclic nucleotide response to PGE<sub>1</sub> over time. The change in platelet cyclic AMP (○) and cyclic GMP (●) with time after stimulation with PGE<sub>1</sub>, expressed as a percentage of basal cyclic nucleotide levels, at the respective time points.

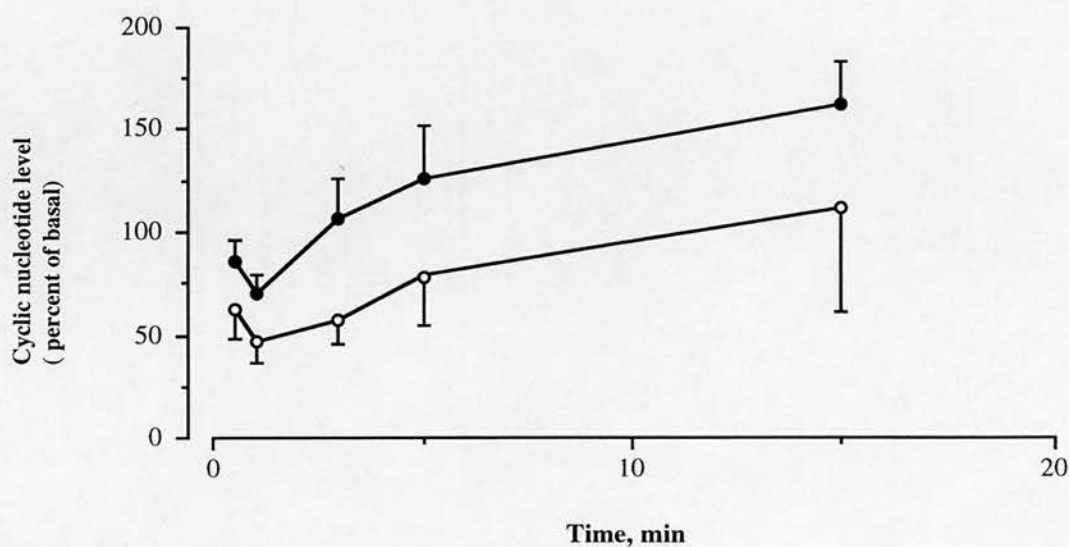


Figure 2.6. Cyclic nucleotide response to SNP over time. The change in platelet cyclic AMP (○) and cyclic GMP (●) with time after stimulation with SNP, expressed as a percentage of basal cyclic nucleotide levels, at the respective time points.

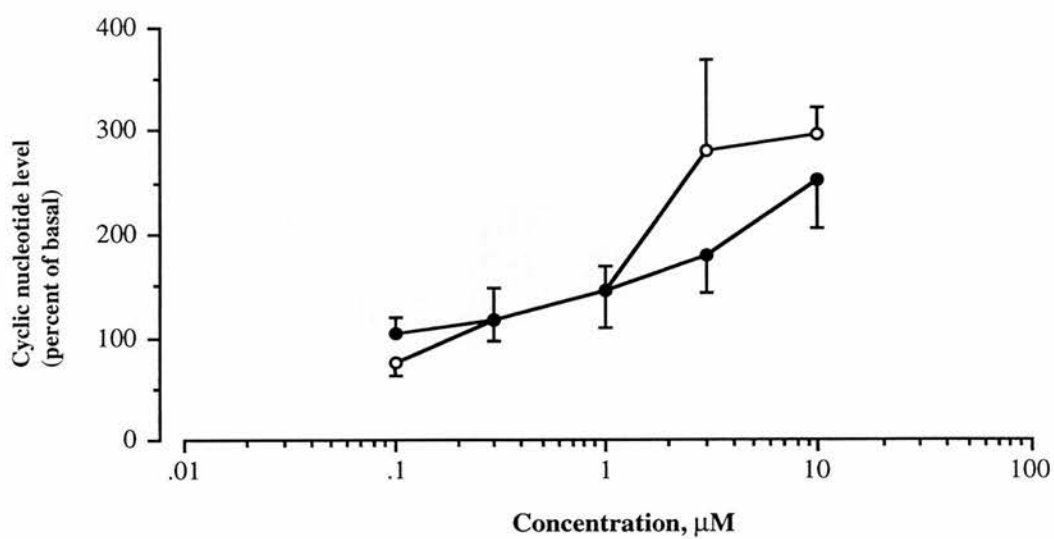


Figure 2.7. Cyclic nucleotide dose response to PGE<sub>1</sub>. PGE<sub>1</sub> stimulated responses in platelet cyclic AMP (○) and cyclic GMP (●).

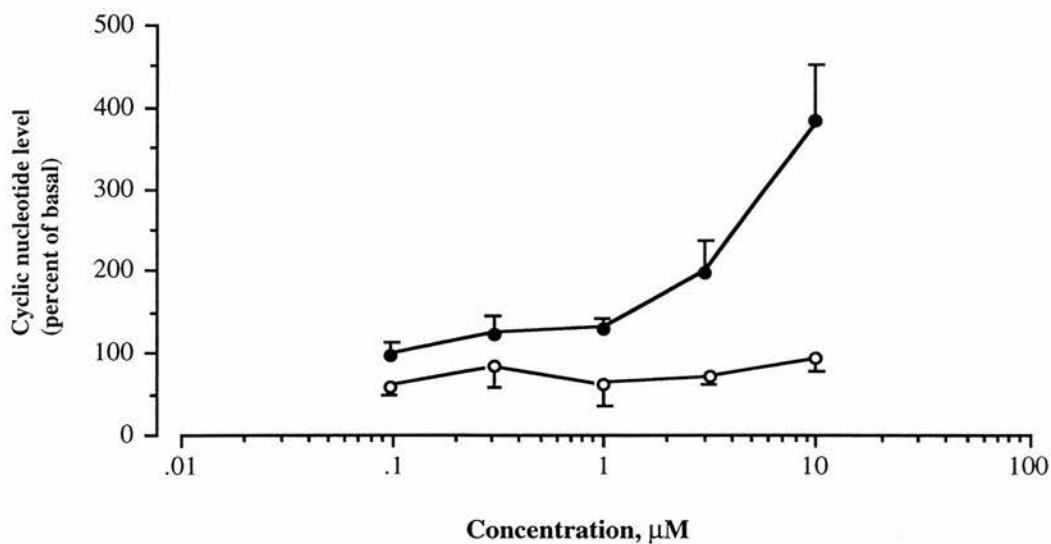


Figure 2.8. Cyclic nucleotide dose response to SNP. SNP stimulated responses in platelet cyclic AMP (○) and cyclic GMP (●).



**APPENDIX 2.1**

**Materials and equipment used.**

**Aggregation**

Anticoagulant

Citric acid	Sigma	Poole, UK.
Glucose	Sigma	Poole, UK
Sodium Citrate	Sigma	Poole, UK

Platelet aggregating agents

Adenosine diphosphate	Sigma	Poole, UK
Adrenaline hydrochloride	Antigen Pharmaceuticals	Roscrea, Ireland

Endothelin agonists/antagonists

Endothelin-1	Novabiochem	Nottingham, UK
Sarafotoxin S6c	Alexis Corporation	Nottingham, UK
BQ-123	SNPE Neosystem	Croydon, UK
BQ-788	SNPE Neosystem	Croydon, UK

Others

N monomethyl-L-arginine	Sigma	Poole, UK
Lemakalim (BRL 38227)	SmithKline Beecham	Welwyn, UK

Vehicle

NaCl	Sigma	Poole, UK
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*Equipment*

Centrifuges

Junior Bench Top	Gallenkamp
Studie M	Wiffug

## Aggregometer

6 channel Clinical Aggregometer	Malin Electronics
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Micro magnetic bars	Mackay & Lynn
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## Computer Hardware

MacLab analogue digital converter	MacLab Analog Digital Instruments
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Macintosh LC	Macintosh
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## Computer Software

Chart	MacLab Analog Digital Instruments
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Excell	Microsoft Corporation
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## Sundries

LP 2 2ml tubes	Sterilin
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## Cyclic Nucleotide Assay

### Agonists

Prostaglandin E <sub>1</sub>	Sigma	Poole, UK
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Sodium Nitroprusside	Sigma	Poole, UK
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### Assay Buffer

Acetic acid	BDH	Poole, UK
-------------	-----	-----------

Sodium acetate	Sigma	Poole, UK
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Gelatin	Sigma	Poole, UK
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### Tracer Preparation

<sup>125</sup> Iodine	Amersham	Amersham, UK
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Tyrosine methyl esters	Sigma	Poole, UK
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Chloramine T	Sigma	Poole, UK
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Potassium dihydrogen orthophosphate	Sigma	Poole, UK
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Disodium hydrogen orthophosphate	Sigma	Poole, UK
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Sodium Metabisulphate	Sigma	Poole, UK
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Butan-1-ol	BDH	Poole, UK
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Whatman 31 filter paper	Whatman	Maidstone, UK
Propan-1-ol	BDH	Poole, UK
cyclic AMP	Sigma	Poole, UK
cyclic GMP	Sigma	Poole, UK
Acetylation		
Triethylamine	BDH	Poole, UK
Acetic anhydride	BDH	Poole, UK
Charcoal		
Charcoal	Sigma	Poole, UK
Dextran T70	Pharmacia	Milton Keynes, UK

#### *Equipment*

RP X-omat processor	Kodak
Coolspin Centrifuge	MSE
Gamma Counter	LKB Wallac

#### Sundries

Plastic LP3 tubes	Sterilin
Borosilicate Glass, 12 by 75 mm	Sterilin
Kodak X-omat film	Kodak

#### **Binding Studies**

Endothelin-1(human, porcine)	Novabiochem	Nottingham, UK
Yohimbine	Sigma	Poole, UK
NaHCO <sub>3</sub>	Sigma	Poole, UK
Phenylsulphonylfluoride	Sigma	Poole, UK
Trizma Base	Sigma	Poole, UK

Trizma HCl	Sigma	Poole, UK
Tracers		
$^{125}\text{I}$ -ET-1 (2200 Ci / mmol)	Dupont	Stevenage, UK
$^3\text{H}$ -Yohimbine(80 Ci / mmol)	Dupont	Stevenage, UK

#### *Equipment*

Homogeniser	Ystral	
J2 MC High Speed Centrifuge	Beckman	
Filtering equipment		
12 Well Filtering Manifolds	Millipore	
Filters	Whatman	
Minaxi TriCarb Beta counter	Packard	
Gamma counter	LKB Wallac	

#### **RT PCR**

##### Platelet preparation

Sodium Citrate	Sigma	Poole, UK
EDTA	Sigma	Poole, UK
M 199	Life Technologies	Paisley, UK
Percoll	Pharmacia	Uppsala, Sweden
Density marker beads	Sigma	Poole, UK

#### *Equipment*

Coolspin Centrifuge	MSE	
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##### RT PCR and mRNA Extraction

Guanidinium Thiocyanate	Fluka	Marseille, France
Sodium Citrate	Merck	Castelnau France
Sodium N-Lauroylsacrosine	Fluka	Marseille, France

Mercapto ethanol	Merck	Castelnau France
Sodium Acetate	Merck	Castelnau France
Chloroform	Merck	Castelnau France
Isoamyl alcohol	Merck	Castelnau France
Glycogen	Fluka	Marseille, France
Isopropanol	Prolabo	Marseille, France
Hexamer nucleotides	Promega	Madison, USA
HPRI RNase inhibitor	Amersham	Amersham, UK
Reverse Transcriptase buffer	Promega	Madison, USA
Dithiothreitol	Merck	Castelnau France
MMLV Reverse transcriptase	Life Technologies	Gaithersburg, USA
Taq DNA Polymerase	Promega	Madison, USA
Agarose	Merck	Castelnau France
Boric Acid	Merck	Castelnau France

#### *Equipment*

L8-S5 ultra Centrifuge	Beckman
PCR Cycling Trio-thermoblock	Biometra

#### **Forearm Blood Flow**

Aspirin	Boots	Nottingham, UK
Anaesthetic		
Xylocaine Hydrochloride	Astra	Kings Langley
	Pharmaceuticals	UK

#### *Equipment*

Syringe Pump	Welmed
Strain gauge Plethysmograph	Vasculab

Classic II computer

Macintosh

**Endothelin Radioimmunoassay**

Radio immunoassay kit

Nichols Institute

Saffron

(including C<sub>18</sub> Sep-Paks)

Walden, UK

## APPENDIX 2.2

### Recipes for buffers used.

Cyclic nucleotide assay buffer	80 ml, 0.1 M acetic acid
0.05M acetate buffer pH 4.8	120 ml, 0.1 M sodium acetate
at 25 °C	200 ml distilled water
	0.1% w/v Gelatin
Phosphate buffer	6.8 g potassium dihydrogen
0.5M pH 7.4 at 25 °C	orthophosphate
	2.84 g disodium hydrogen
	orthophosphate
	dissolved in 100 ml distilled water
Tris Buffer	6.61 g Trizma HCl
0.5M pH 7.4 at 25 °C	0.97 g Trizma Base
	1 L distilled water
Activated Charcoal	1 L 0.1M phosphate buffer
	(as described above)
	60 g charcoal
	6 g dextran T70
Solution D	4 M guanidinium thiocyanate
for RNA extraction	25 mM sodium citrate
pH 7.0 at 25 °C	0.5% v/v sodium N-Lauroylsacrosine
	solution (30% in H <sub>2</sub> O)
	0.7% v/v mercapto ethanol



TBE Buffer

108.9 g Tris

55.7 g boric Acid

4.7 g EDTA

1 L distilled water

## **CHAPTER 3**

### **INVESTIGATION INTO THE EFFECTS OF ENDOTHELIN-1 ON PLATELET AGGREGATION**

## INTRODUCTION

At the time of this work being carried out, studies on the actions of ET-1 on platelets had consistently reported that ET-1 alone does not induce platelet aggregation. However, it was reported that ET-1 potentiated aggregation of human platelets induced by low concentrations of adrenaline *in vitro* (Matsumoto, *et al.*, 1990). An inhibition of platelet aggregation was also reported in a study of *ex vivo* platelet aggregation after *in vivo* administration of ET-1 in rabbits (Thiemermann, *et al.*, 1989). Platelet responses following *in vivo* administration of ET-1 involve a number of other factors as well as a direct action of ET-1 itself, the possible role of endothelium derived mediators is discussed later in this thesis. The phases of primary and secondary aggregation observed *in vitro* in response to weak agonists represent two distinct mechanisms. Primary aggregation is thought to represent the direct receptor mediated response to the agonist and the subsequent intracellular signalling. Secondary aggregation represents a response dependent on the positive 'feed forward' actions of intracellular signalling and the release of other pro-aggregatory agents such as serotonin and ADP. Hence, the apparently contradictory potentiating and inhibitory actions of ET-1 may be due to the peptide interacting in different manners with the two separate processes involved in platelet aggregation.

The mechanism of ET-1 mediated actions are not well understood. Initially it was proposed that ET-1 might be an endogenous agonist of the dihydropyridine-sensitive voltage operated calcium channels (Goto *et al.*, 1989), subsequent evidence suggested that this was not the case (Gu, *et al.*, 1989). However, there is considerable evidence for an interaction with ATP-sensitive K<sup>+</sup> channels. K<sup>+</sup> channel openers inhibited ET-1 induced contractions in rat arterial vessels (O'Donnell *et al.*, 1990) and in the human hand vein (Haynes & Webb, 1993c). Furthermore, the ATP-sensitive K<sup>+</sup> channel opener BRL 38227 inhibited the binding

of  $^{125}\text{I}$ -ET-1 to rat cardiac membranes, indicating a possible relationship between ET-1 receptor binding and  $\text{K}^+$  channels (Waugh, *et al.*, 1992).

The endothelial  $\text{ET}_\text{B}$  receptor mediates NO generation by vascular endothelial cells (Emori *et al.*, 1991). NO is known to bring about relaxation of vascular smooth muscle cells and the inhibition of platelet aggregation (Moncada & Higgs, 1993). In 1990, Radomski *et al* characterised an L-arginine/NO pathway in human platelets and suggested it may have a role as a negative feedback mechanism for inhibiting platelet aggregation (Radomski, *et al.*, 1990). NO production is competitively inhibited by L-NMMA and it can, therefore, be used to investigate the effects of NO synthesis. In the absence of an accessible, sensitive method of directly measuring NO, L-NMMA has been employed to identify NO synthase activity.

The aim of the work presented in this chapter was (a) to investigate the direct actions of ET-1 on human platelet aggregation and (b) the ability of ET-1 to modulate primary and secondary aggregation induced by the two physiological platelet activators, adrenaline and ADP. BRL 38227 and L-NMMA were employed in an attempt to identify mechanisms of action of ET-1.

## **METHODS**

The work described took the form of three separate studies. Study A was designed to investigate the actions of ET-1 alone and in combination with doses of ADP and adrenaline that elicited primary and secondary aggregation. Study B examined the effect of BRL 38227 on ET-1 modulation of adrenaline induced aggregation, and in study C, L-NMMA was used to investigate whether ET-1-induced platelet NO production masked either a direct aggregating effect of ET-1 or attenuated ET-1 mediated potentiation of adrenaline induced aggregation.

## **Subjects**

Healthy male subjects between 21 and 41 years of age participated in these studies. All subjects complied to conditions stated in chapter 2, that is that no subject received vasoactive or non-steroidal anti inflammatory drugs in the ten days before each study or alcohol or caffeine on the day of the studies and all subjects rested supine for 30 min in a quiet environment prior to venesection. These conditions were maintained for all aggregation studies reported in this thesis unless otherwise stated.

For all these studies blood was collected into acid citrate dextrose via a 19 g needle and plasma was prepared as described in chapter 2. Platelet rich plasma was divided into 890 µl aliquots and incubated at 37°C for 3 min prior to any additions. Platelet aggregation was measured by Born aggregometry also as described in chapter 2, and all studies were carried out within 150 min of venesection. Results are expressed as mean  $\pm$  s.e.mean and statistical significance was established using a paired Student's t-test.

## **Study protocols**

### *Study A*

Aggregatory responses to ADP and adrenaline were assessed for each subject to establish appropriate doses of the aggregating agent to induce primary and secondary aggregation. Subsequently, the effect of pre-incubation with ET-1, 1 µM, was measured alone, and in combination with doses of adrenaline and ADP which induced primary and secondary aggregation (table 3.1).

Fourteen volunteers gave blood on two separate occasions. On one occasion the effects of ET-1 on adrenaline-induced aggregation were tested and on the other the effects on ADP-induced aggregation were tested. In table 3.1 the sequence of additions to each tube are given for the three parts of the study carried out on every subject, adrenaline is given as the aggregating agent but the same protocol was used for ADP. Although the sequence of additions to each tube were always the same, the order of the tubes, 1, 2, 3 or 4 first for example was randomised.

### *Study B*

Before investigating the effect of BRL 38227 on ET-1 modulated platelet aggregation, the effect of BRL 38227, 1 $\mu$ M, was examined alone and in combination with doses of adrenaline inducing primary and secondary aggregation. The effect of pre-incubation with BRL 38227 was then investigated in combination with adrenaline and ET-1 (table 3.2a and 3.2b). Two groups of ten healthy male volunteers were studied.

	Tube 1	Tube 2	Tube 3	Tube 4
Run 1	Vehicle	Vehicle	Vehicle	Vehicle
	Vehicle	Vehicle	Vehicle	Vehicle
	Adrenaline	Adrenaline	Adrenaline	Adrenaline
Run 2	Vehicle	Vehicle	Vehicle	Vehicle
	Vehicle	Vehicle	ET-1	ET-1
Primary Aggregation	Vehicle	Adrenaline/ ADP	Adrenaline/ ADP	Vehicle
Run 3	Vehicle	Vehicle	Vehicle	Vehicle
	Vehicle	Vehicle	ET-1	ET-1
Secondary Aggregation	Vehicle	Adrenaline/ ADP	Adrenaline/ ADP	Vehicle

Table 3.1



	Tube 1	Tube 2	Tube 3	Tube 4
Run 1	Vehicle	Vehicle	Vehicle	Vehicle
	Vehicle	Vehicle	Vehicle	Vehicle
	Adrenaline	Adrenaline	Adrenaline	Adrenaline
Run 2	Vehicle	Vehicle	BRL 38227	BRL 38227
	Vehicle	Vehicle	Vehicle	Vehicle
Primary Aggregation	Vehicle	Adrenaline	Adrenaline	Vehicle
Run 3	Vehicle	Vehicle	BRL 38227	BRL 38227
	Vehicle	Vehicle	Vehicle	Vehicle
Secondary Aggregation	Vehicle	Adrenaline	Adrenaline	Vehicle

Table 3.2a

	Tube 1	Tube 2	Tube 3	Tube 4
Run 1	Vehicle	Vehicle	Vehicle	Vehicle
	Vehicle	Vehicle	Vehicle	Vehicle
	Adrenaline	Adrenaline	Adrenaline	Adrenaline
Run 2	Vehicle	Vehicle	Vehicle	BRL 38227
	Vehicle	Vehicle	ET-1	ET-1
	Vehicle	Adrenaline	Adrenaline	Vehicle
Run 3	Vehicle	Vehicle	Vehicle	BRL 38227
	Vehicle	Vehicle	ET-1	ET-1
	Vehicle	Adrenaline	Adrenaline	Vehicle
Secondary Aggregation	Vehicle	Vehicle	Vehicle	BRL 38227
	Vehicle	Vehicle	ET-1	ET-1
	Vehicle	Adrenaline	Adrenaline	Vehicle

Table 3.2b

*Study C*

To ascertain whether platelet derived NO masked a direct aggregating effect of ET-1 on human platelets, the effect of pre-incubation of 10 & 100  $\mu$ M L-NMMA, prior to ET-1 (1  $\mu$ M) exposure was examined (table 3.3a). To determine whether ET-1 induced NO attenuated the potentiation of adrenaline induced primary aggregation, L-NMMA (100 $\mu$ M) and ET-1 (1 $\mu$ M) were added to the PRP prior to adrenaline. (Table 3.3b).

	Tube 1	Tube 2	Tube 3	Tube 4
Run 1	Vehicle	Vehicle	Vehicle	Vehicle
	Vehicle	Vehicle	L-NMMA 10 $\mu$ M	L-NMMA 10 $\mu$ M
	Vehicle	ET-1	ET-1	Vehicle
Run 2	Vehicle	Vehicle	Vehicle	Vehicle
	Vehicle	Vehicle	L-NMMA 100 $\mu$ M	L-NMMA 100 $\mu$ M
	Vehicle	ET-1	ET-1	Vehicle

Table 3.3a

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
Run 1	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle
	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle
	Adrenaline	Adrenaline	Adrenaline	Adrenaline	Adrenaline	Adrenaline
Run 2	Vehicle	Vehicle	Vehicle	L-NMMA	L-NMMA	L-NMMA
	Vehicle	Vehicle	ET-1	ET-1	Vehicle	ET-1
	Vehicle	Adrenaline	Adrenaline	Adrenaline	Adrenaline	Vehicle
Run 3	Vehicle	Vehicle	Vehicle	L-NMMA	L-NMMA	L-NMMA
	Vehicle	Vehicle	ET-1	ET-1	Vehicle	ET-1
	Vehicle	Adrenaline	Adrenaline	Adrenaline	Adrenaline	Vehicle

Table 3.3b

## RESULTS

### Study A

ET-1 alone caused a slight but significant aggregatory response ( $3.8 \pm 0.4\%$ ) when compared to vehicle ( $2.7 \pm 0.2$ ) ( $p=0.04$ ,  $n = 14$ ). No shape change was observed.

Both adrenaline and ADP induced dose dependent platelet aggregation (Figs 3.1 & 3.2). Pre-incubation with ET-1 for 1 min significantly potentiated adrenaline-induced primary aggregation, ( $15.7 \pm 3.1\%$  adrenaline alone vs  $25.5 \pm 6.6\%$  adrenaline + ET-1;  $p = 0.04$ ,  $n=14$ ), and significantly inhibited secondary aggregation, ( $70.3 \pm 4.2\%$  adrenaline alone vs  $61.5 \pm 7.3\%$  adrenaline + ET-1;  $p = 0.04$ ,  $n=14$ )(Fig 3.3). The effect of ET-1 did not appear uniform for all subjects, as indicated by the dispersion of the data seen with ET-1. ADP induced primary and secondary aggregations were not significantly affected by pre-incubation with ET-1 (Fig 3.3).

### Study B

BRL 38227 alone did not induce platelet aggregation and adrenaline induced primary and secondary aggregation were not significantly affected by pre-incubation with BRL 38227.

The pre-incubation with ET-1 again significantly potentiated adrenaline induced primary aggregation, ( $9.7 \pm 1.1\%$  adrenaline alone vs  $25.5 \pm 6.6\%$  adrenaline + ET-1;  $p = 0.04$ ,  $n=10$ ), but pre-incubation with BRL 38227 did not significantly alter this effect (fig 3.4).

ET-1 tended to inhibit secondary aggregation however this did not reach statistical significance (adrenaline alone  $68.4 \pm 7.0\%$ , ET-1 + adrenaline  $55.6 \pm 13.8\%$ ;  $p=0.18$ ,  $n=10$ ). BRL 38227 had no significant effect on secondary aggregation in the presence of ET-1 (fig 3.4).

### Study C

There was no significant difference in the response to ET-1 whether or not L-NMMA (1 nM & 1 $\mu$ M) was present.

Primary aggregation to adrenaline was studied at two concentrations for each subject; a lower concentration chosen to achieve very low level aggregation, threshold concentration, and a higher concentration, to attain moderate primary but not secondary aggregation. At the threshold concentration, ET-1 potentiated the adrenaline response, and L-NMMA produced a still greater response, (aggregation to adrenaline alone  $5.5 \pm 0.7\%$ , adrenaline + ET-1  $18.2 \pm 5.6\%$ ,  $p=0.04$ ,  $n=12$ , adrenaline + ET-1 + L-NMMA  $39.0 \pm 9.6\%$ , vs adrenaline alone  $p=0.01$ , vs adrenaline + ET-1  $p=0.05$ ) (fig 3.5). Pre-incubation with L-NMMA alone significantly potentiated aggregation induced by threshold concentrations of adrenaline ( $5.5 \pm 0.7\%$  vs  $32.2 \pm 8.4\%$ ,  $p=0.01$   $n=12$ ). However there was no difference between the aggregation to adrenaline with L-NMMA whether ET-1 was present or not (fig 3.5).

At the higher concentration of adrenaline, ET-1 again potentiated the adrenaline-induced aggregation, (adrenaline  $10.6 \pm 1.9\%$ , adrenaline + ET-1  $20.4 \pm 6.7\%$ ;  $p=0.05$   $n=12$ ). However, L-NMMA had no significant effect in the presence or absence of ET-1 (fig 3.5).

## DISCUSSION

The results of these studies demonstrate that ET-1 slightly, but significantly increases light transmittance through PRP, indicating a very weak aggregatory effect. However, this direct effect was small and is probably not of physiological significance. ET-1 potentiated adrenaline-induced primary aggregation, and tended to inhibit adrenaline-induced secondary aggregation. However, ADP-induced aggregation was unaffected by ET-1. ET-1 potentiation of adrenaline-induced aggregation was unaffected by the ATP-sensitive K<sup>+</sup> channel opener BRL 38227, indicating that ATP-sensitive K<sup>+</sup> channels are not involved in ET-1 mediated effects on adrenaline-induced aggregation of human platelets. Pre-incubation with the NO synthase inhibitor L-NMMA does not reveal a greater effect of ET-1 alone. The potentiation of low level platelet aggregation to adrenaline by ET-1 was further increased by LNMMA. However, this is not significantly greater than the effect of LNMMA on adrenaline-induced aggregation in the absence of ET-1. Therefore, ET-1 does not appear to stimulate NO synthase to a degree that inhibits platelet aggregation.

ET-1, at threshold concentrations, is known to potentiate the vasoconstrictor actions of other agonists, notably noradrenaline and serotonin (Yang, *et al.*, 1990). The potentiation of primary aggregation by ET-1 in platelets appears to be dependent on the aggregating agent employed. The lack of potentiation of ADP-induced primary aggregation suggests that ET-1 either directly interacts with the adrenergic  $\alpha_2$  receptors, the receptor mediating the aggregatory actions of adrenaline, or acts upon an intracellular pathway associated with adrenaline but not ADP platelet activation. Protein kinase C is linked to  $\alpha_2$  receptor activation on human platelets, but may only play a part in ADP-induced secondary aggregation (Nieuwland, *et al.*, 1993). Since



ET-1 is known to stimulate protein kinase C in other cells (Lee, *et al.*, 1989) this enzyme may be a possible target for ET-1 in platelets. The inhibition of secondary aggregation by ET-1 did not achieve the same statistical significance in study B as it did in study A, though the trend was the same, and it is possible that this effect may be more clearly defined if a 'strong' platelet aggregating agent, capable of directly eliciting secondary aggregation such as thrombin, were to be used.

The fact that BRL 38227 did not affect ET-1 potentiation of aggregation suggests that the mechanism of action of ET-1 in platelets differs from that seen in other cells types such as rat cardiac cells. The ATP sensitive K<sup>+</sup> channel opener pinacidil has been shown to inhibit *in vitro* platelet aggregation to ADP by the attenuation of thromboxane generation, suggesting a role for K<sup>+</sup> channels in the modulation of platelet aggregation (Goodman *et al.*, 1985) In the work presented here, BRL 38227 (1 µM) did not modulate platelet primary or secondary aggregation to adrenaline in the presence or absence of ET-1. This is consistent with a study published in 1994, which showed no effect on adrenaline-induced aggregation of pinacidil at 100 µM (Patelunas Hoffman *et al.*, 1994). However, in the study reported by Goodman pinacidil was used at 10<sup>-4</sup> to 10<sup>-2</sup> M, which is by their own admission several orders of magnitude higher than the serum concentration required for antihypertensive effects and it is likely that Goodman observed an effect not directly related to platelet potassium channels.

In study C, L-NMMA did not significantly alter the effect of ET-1 alone or in the presence of adrenaline suggesting that NO inhibition in platelets does not unmask a direct aggregatory response to ET-1. An interesting result of study C was the potentiation by L-NMMA of primary aggregation induced by threshold concentrations of adrenaline but not higher concentrations. This might suggest that platelet derived NO is more important in the modulation of low level platelet



aggregation, and when a stronger intracellular signal is elicited NO is of less importance. There is some controversy over the importance of platelet derived NO in attenuating platelet aggregation. NO production has been measured directly, by the use of a microsensor from platelets stimulated with collagen (Malinski *et al.*, 1993). However, it has been suggested that platelets do not produce sufficient NO to attenuate the aggregatory response to all agonists (Mollace *et al.*, 1991). In the original description of the L-arginine:NO pathway in platelets L-arginine only inhibited thrombin induced platelet aggregation in the presence of phosphodiesterase inhibitors (Radomski, *et al.*, 1990). The inhibitory actions of cyclic GMP, the second messenger of NO are thought to be mediated by a decrease in  $[Ca^{2+}]_i$ . Increased  $[Ca^{2+}]_i$  is not thought to play a major role in adrenaline-induced platelet activation, although a slight rise is detectable using the luminescent calcium sensitive dye aequorin (Powling & Hardisty, 1987). It may be that at low concentrations of adrenaline the slight rise in  $[Ca^{2+}]_i$  is functionally important but that at higher concentrations of agonist an alternative intracellular mechanism supercedes the slight calcium effect.

In conclusion this chapter provides evidence for ET-1 potentiation of adrenaline induced primary aggregation that is independent of ATP-sensitive  $K^+$  channels and not attenuated by ET-1 stimulation of NO synthase. ET-1 also appears to inhibit adrenaline-induced secondary aggregation.

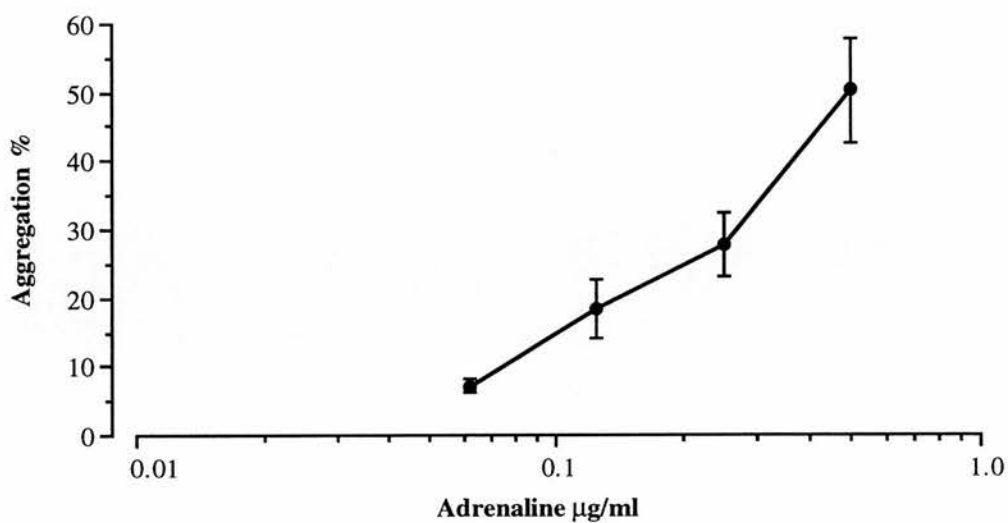


Figure 3.1 Dose dependent platelet aggregation induced by adrenaline, mean  $\pm$  s.e.mean, n=14.

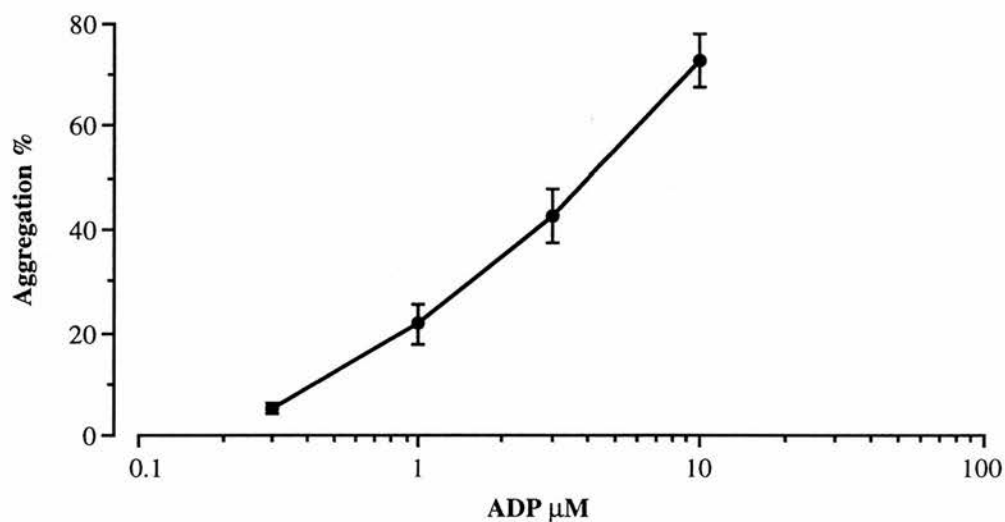


Figure 3.2 Dose dependent platelet aggregation induced by ADP, mean  $\pm$  s.e.mean, n=14.

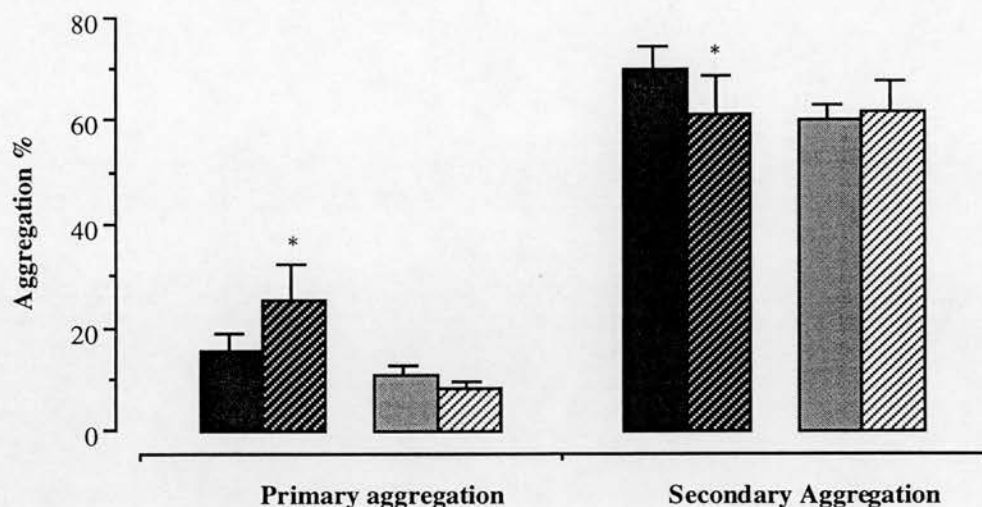


Figure 3.3. The effect of ET-1 on adrenaline and ADP induced primary and secondary aggregation, mean  $\pm$  s.e.mean. Adrenaline-induced primary aggregation ■ was significantly potentiated by pre-incubation with ET-1 ▨ ,  $p = 0.04$ , and adrenaline-induced secondary aggregation ■ was significantly inhibited by ET-1 ▨  $p = 0.04$ . ADP induced primary and secondary aggregation ■ was not significantly affected by incubation with ET-1 ▨ . Statistical analysis was by paired Student's  $t$  test,  $n=14$  for all comparisons.

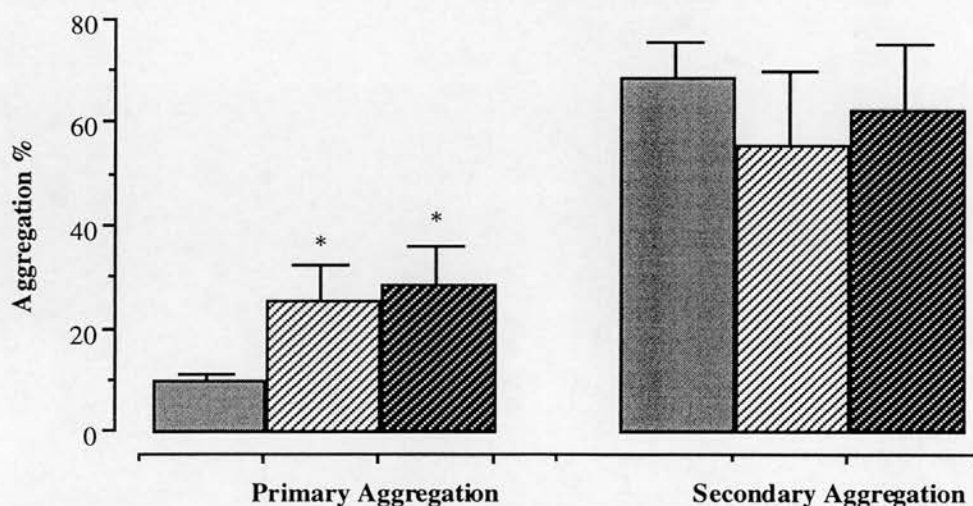


Figure 3.4. Aggregation to adrenaline ■ , adrenaline + ET-1 ▨ and adrenaline + ET-1 + BRL 38227 ■ , mean  $\pm$  s.e.mean, \*  $p \leq 0.05$  compared to adrenaline alone. Adrenaline-induced primary aggregation was significantly potentiated by ET-1,  $p = 0.04$ , this effect was unaltered by BRL 38227. Secondary aggregation to adrenaline was not significantly altered by pre-incubation with either ET-1 or BRL 38227.

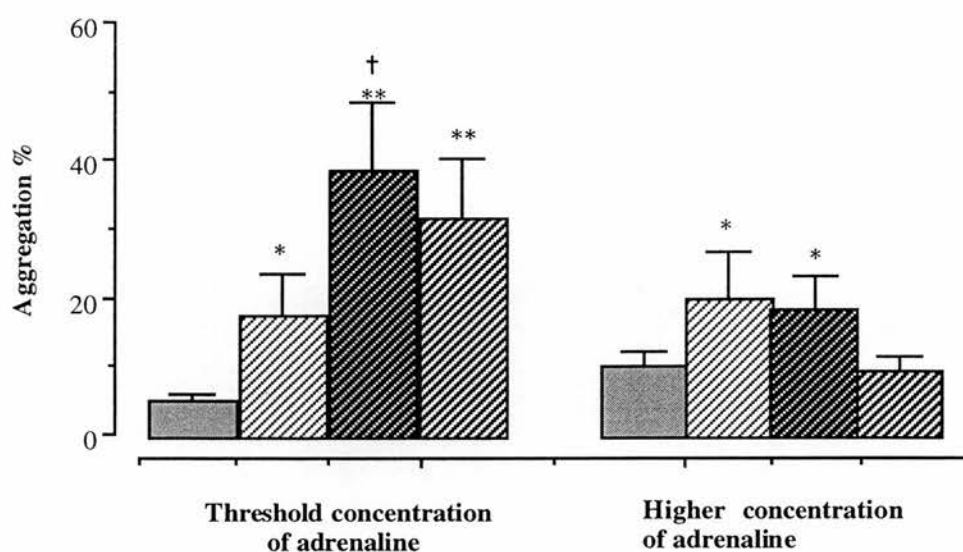






Figure 3.5. The effect of ET-1 and L-NMMA on threshold, and high primary aggregation to adrenaline; adrenaline alone , adrenaline + ET-1 , adrenaline + ET-1 + L-NMMA  and adrenaline + L-NMMA , \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$  vs adrenaline alone, †  $p \leq 0.05$  vs adrenaline + ET-1, mean  $\pm$  s.e.mean. ET-1 potentiated both levels of platelet aggregation, L-NMMA further potentiated the effect of ET-1 on the threshold concentration of adrenaline, but this was not significantly different from the effect of L-NMMA alone on adrenaline.

## **CHAPTER 4**

### **CHARACTERISATION OF THE RECEPTOR ON PLATELETS WHICH MEDIATES ENDOTHELIN-1 INDUCED POTENTIATION OF AGGREGATION**

## INTRODUCTION

ET-1 acts on cell surface G protein linked receptors. To date, two receptors have been cloned from mammalian tissue, the ET<sub>A</sub> and ET<sub>B</sub> receptors (Arai, *et al.*, 1990; Sakurai, *et al.*, 1990). In the human vasculature the ET<sub>A</sub> receptor is found primarily on smooth muscle cells. It preferentially binds ET-1 with relative affinity for the ETs of ET-1>ET-2>>ET-3. The ET<sub>B</sub> receptor was initially thought to be primarily located on the endothelial cells, where it mediates relaxation through the release of prostacyclin and NO. The ET<sub>B</sub> receptor is isopeptide non-selective having approximately equal affinity for all three ETs (Haynes & Webb, 1993a). Subsequent work has identified ET<sub>B</sub> receptors on vascular smooth muscle cells mediating contraction (Clozel, *et al.*, 1992; Douglas, *et al.*, 1995).

The snake venom peptide, sarafotoxin 6c (SRTX S6c), a peptide of similar structure to the ETs, has been demonstrated to be a selective ET<sub>B</sub> receptor agonist (Williams, *et al.*, 1991) inducing vasodilatation (Kloog & Sokolovsky, 1989) and constriction (Haynes *et al.*, 1995). It is, at least, a thousand fold less potent than ET-1 in activating ET<sub>A</sub> receptors to produce a functional response in the rat vasculature (Williams, *et al.*, 1991).

A number of selective antagonists have been developed for both ET<sub>A</sub> and ET<sub>B</sub> receptors including antagonists that distinguish putative functionally different ET<sub>B</sub> receptors (Warner *et al.*, 1993). The pentapeptide BQ-123 is a selective antagonist at the ET<sub>A</sub> receptor, inhibiting both ET-1 binding and ET-1 induced contraction (Ihara, *et al.*, 1992). IC<sub>50</sub> values for BQ-123 have been calculated as 7.3 nM at the ET<sub>A</sub> receptor and 18 µM at the ET<sub>B</sub> receptor, in rat vascular smooth muscle and cerebellum respectively (Ihara, *et al.*, 1992). The antagonist BQ-788 is selective for ET<sub>B</sub> receptor, acting as an antagonist at both of the proposed sub -types of the ET<sub>B</sub>

receptor (Karaki, *et al.*, 1994). IC<sub>50</sub> values for BQ-788 have been calculated as 1.3  $\mu$ M at the ET<sub>A</sub> receptor and 0.9 nM at the ET<sub>B</sub> receptor in a human neuroblastoma cell line and porcine cerebellum respectively (Ishikawa, *et al.*, 1994).

The work described here was designed to investigate the ET receptor that mediates the potentiation of adrenaline-induced aggregation described in the previous chapter. It may be useful to briefly consider what is currently known about ET receptors on platelets in general. A number of groups have investigated the ET receptor mediated inhibition of platelet aggregation. A recent report looking at a number of different platelet aggregating agents, showed that ET-1 inhibited platelet aggregation to thrombin, ADP, collagen and noradrenaline (Touyz & Schiffrin, 1995). However, characterisation of the receptor involved, by the use of various agonists and antagonists, has produced inconclusive results. Astarie-Dequeker (Astarie-Dequeker *et al.*, 1992) observed an equal inhibition by ET-1 and ET-3 of thrombin-induced aggregation *in vitro*, suggesting that the ET<sub>B</sub> receptor may be mediating this effect. However, Touyz reported a dose dependent inhibition of platelet aggregation and an increase in platelet pH<sub>i</sub> by ET-1; the latter blocked by BQ-123. Touyz inferred that the ET<sub>A</sub> receptor mediated inhibition of aggregation (Touyz & Schiffrin, 1993b). It would appear, therefore, that platelets express ET<sub>A</sub> and ET<sub>B</sub> receptors and that ET-1 inhibition of aggregation may be mediated by both receptor sub-types.

The work described in this chapter consists of three separate studies: the first examined the effects of receptor agonists, ET-1 and SRTX S6c on adrenaline-induced primary aggregation; the second assessed whether the effect of ET-1 was reproducible on different aliquots of plasma from the same subjects (that is whether repeated aggregation studies would produce similar results); and the third examined the effect of pre-incubation with the selective antagonists BQ-123 and BQ-788 on ET-1 mediated potentiation of primary platelet aggregation.



## Methods

### Subjects:

Healthy male subjects between the ages of 21 and 41 years of age participated in these studies.

Preparation of PRP from venous blood and platelet aggregation studies were carried out as described in chapter 2. Briefly, blood was taken via a 19 gauge needle into acid citrate dextrose; 9:1. Blood was promptly centrifuged at room temperature for 10 min at 120 *g* to obtain PRP and the remaining blood was centrifuged for a further 20 min at 3000 *g* to obtain PPP. PRP was transferred carefully into 2 ml tubes and stored under 95% O<sub>2</sub> 5% CO<sub>2</sub> at room temperature until required. Samples were heated to 37°C and stirred at 1200 rpm and allowed to equilibrate for 3 min prior to each study.

In all studies aggregatory responses to adrenaline were assessed for each subject to establish appropriate doses of the aggregating agent to induce primary aggregation.

### Study 1

Samples were pre-incubated with ET-1 ( $10^{-12}$  to  $10^{-9}$  M), SRTX S6c ( $10^{-12}$  to  $10^{-9}$  M) or vehicle (normal saline) for 1 min prior to the addition of either adrenaline, at the individually pre-determined concentration or vehicle. The order of addition, that is whether ET-1 or SRTX S6c was used first, was randomised.

### Study 2

Samples were pre-incubated with ET-1 ( $10^{-12}$  to  $10^{-9}$  M) or vehicle for 1 min prior to the addition of adrenaline or vehicle. In samples from individuals displaying a

potentiation to adrenaline (an increase of 5% or more compared to adrenaline alone at two concentrations of ET-1) the dose response to ET-1 was repeated.

### Study 3

Samples were pre-incubated with ET-1 ( $10^{-12}$  to  $10^{-9}$  M) or vehicle for 1 min prior to the addition of adrenaline or vehicle. In samples from individuals displaying a potentiation to adrenaline, as described for study 2, samples were then incubated with BQ-123 1 $\mu$ M or BQ-788 0.1  $\mu$ M for 1 min prior to the addition of ET-1 ( $10^{-12}$  to  $10^{-9}$  M) or vehicle and after a further minute the predetermined dose of adrenaline was added.

### Statistical Analysis

Results are expressed as mean  $\pm$  s.e.mean, statistical analysis was by repeated measure ANOVA. Differences are considered to be statistically significant at the 5% level. For comparisons giving a significant result by ANOVA the Fisher test was used to identify individual points that were significantly different from adrenaline alone for study 1 or ET treatment in the absence of an antagonist for study 3.

## Results

Pre-incubation with ET-1 ( $10^{-12}$ - $10^{-9}$ ) modestly but significantly potentiated adrenaline induced aggregation ( $p=0.03$ ,  $n=12$ ). The response to SRTX S6c and adrenaline was more variable with mean standard deviations approaching double those of ET-1, SRTX S6c potentiation of adrenaline-induced aggregation was of borderline significance ( $p=0.05$ ,  $n=12$ ) (fig 4.1). A comparison of the effect of both agonists at the maximum concentration,  $10^{-9}$  M, reveals a positive relationship, with the exception of one marked outlier (fig 4.1b). In general, subjects displaying little or no potentiation of primary aggregation by ET-1 display little or no potentiation by SRTX S6c, and similarly, with the exception of the one outlier, subjects displaying a greater effect, do so to both agonists. However, at this concentration of agonist the effect of SRTX S6c is greater than that of ET-1 for all subjects. Even subjects showing little or no potentiation to ET-1 appear to show a greater potentiating effect to SRTX S6c.

When a dose response to ET-1 was repeated in plasma from subjects in whom potentiation of adrenaline-induced aggregation was observed ET-1 again significantly potentiated primary aggregation ( $p=0.02$ ,  $n=6$ ). There was no significant difference between the first and second ET-1 challenge ( $p=0.86$ ,  $n=6$ ) (fig 4.2).

A one minute incubation with BQ-123,  $10^{-6}$  M, did not significantly alter the potentiation of aggregation by ET-1 ( $p=0.88$ ,  $n=6$ ) (fig 4.3). However, BQ-788,  $10^{-7}$  M, did significantly attenuate the ET-1 mediated response ( $p=0.04$   $n=6$ ) (fig 4.4). Neither antagonist had any effect on platelet aggregation alone or in the presence of adrenaline.

## Discussion

In platelets from healthy control subjects, adrenaline-induced *in vitro* primary platelet aggregation was potentiated in a dose dependent manner by ET-1 and SRTX S6c. The potentiation was neither universal nor uniform. Potentiation to SRTX S6c was particularly variable as demonstrated by the large s.e.means seen in figure 4.1. In figure 4.1b the subjects appear to fall into 2 clusters, with the exception of the outlier, those who show a potentiation to ET-1 and those who show no effect. This would be consistent with the proposition by Matsumoto that some individuals display potentiation of aggregation by ET-1 and some do not (Matsumoto, *et al.*, 1990). However, in the study presented here the difference between these groups is relatively small compared to the variability among the responders. Consequently, in any study designed to directly test the proposition that a bimodal distribution of response exists a greater number of subjects would be required.

Study 2 was designed to test whether the potentiation of aggregation by ET-1 was reproducible. Separate aliquots of plasma from individuals showing a response were subjected to the same doses of ET-1 as in study 1. A similar profile of responses was observed, and consequently potentiation of aggregation by ET-1 was considered to be reproducible. The aim of this work was to characterise the receptor mediating the potentiation of adrenaline-induced primary aggregation by ET-1 therefore the antagonist studies were only performed in subjects exhibiting potentiation of aggregation by ET-1. These subjects are referred to hereafter as 'responders'.

In 'responders' (those subjects showing an increase of 5% or more compared to adrenaline alone at two concentrations of ET-1) the effect of ET-1 was not antagonised by the ET<sub>A</sub> selective antagonist BQ-123 but was significantly attenuated by the ET<sub>B</sub> selective antagonist BQ-788. This indicates that the ET<sub>A</sub> receptor does

not play a significant role in potentiation of adrenaline-induced primary aggregation by ET-1 and that the major contribution is mediated by the ET<sub>B</sub> receptor.

In figures 4.2, 4.3 and 4.4 the aggregatory response to adrenaline alone appears to be higher than that observed in study 1, shown in figure 4.1. This raises the questions of whether the higher initial extent of aggregation is responsible for the greater potentiation by ET-1. This is answered, to a certain extent, by the result described in chapter 3 where the potentiating effect of ET-1 was at least as great, if not greater, when a threshold concentration of adrenaline was used compared to a higher concentration.

The term 'responder' is used to describe the effect observed in this study. Although this is consistent with the proposition of Matsumoto, discussed above, from these studies it is not possible to establish an intrinsic difference between subjects. Platelet activity is affected by a number of environmental factors, such as diet, exercise and stress, and although attempts were made to control for some of these it is still possible that activity and responses of platelets may differ from day to day. To establish whether there are subgroups of responders, it would not only be useful to study a large group of subjects but also to study subjects repeatedly on several occasions.

As mentioned in the introduction to this chapter Touyz and Schiffrin described a dose dependent increase in platelet pH<sub>i</sub> by ET-1. Although these researchers suggested that the alkalisation was associated with an inhibition of aggregation, they also demonstrated that alkalisation induced by angiotensin II correlated with an increase in intracellular Ca<sup>2+</sup> (Touyz & Schiffrin, 1993b). It is, therefore, possible that the alkalisation reported may be associated with a pro-aggregatory effect. However, in the work presented in this chapter no effect of the ET<sub>A</sub> receptor

antagonists BQ-123 on ET-1 potentiation of aggregation in the 'responder' group was observed, suggesting this is not mediated by the BQ-123 inhibitable increase in  $pH_i$  described by Touyz.

In conclusion, the potentiation of adrenaline induced aggregation by ET receptor agonists appears to be variable between subjects but reproducible in individual subjects. This pro-aggregatory effect is observed with SRTX S6c as well as ET-1, and the actions of ET-1 are inhibited by BQ-788 but not BQ-123. This indicates that platelets from at least some individuals possess  $ET_B$  receptors and that these receptors mediate ET-1 potentiation of adrenaline induced primary aggregation.

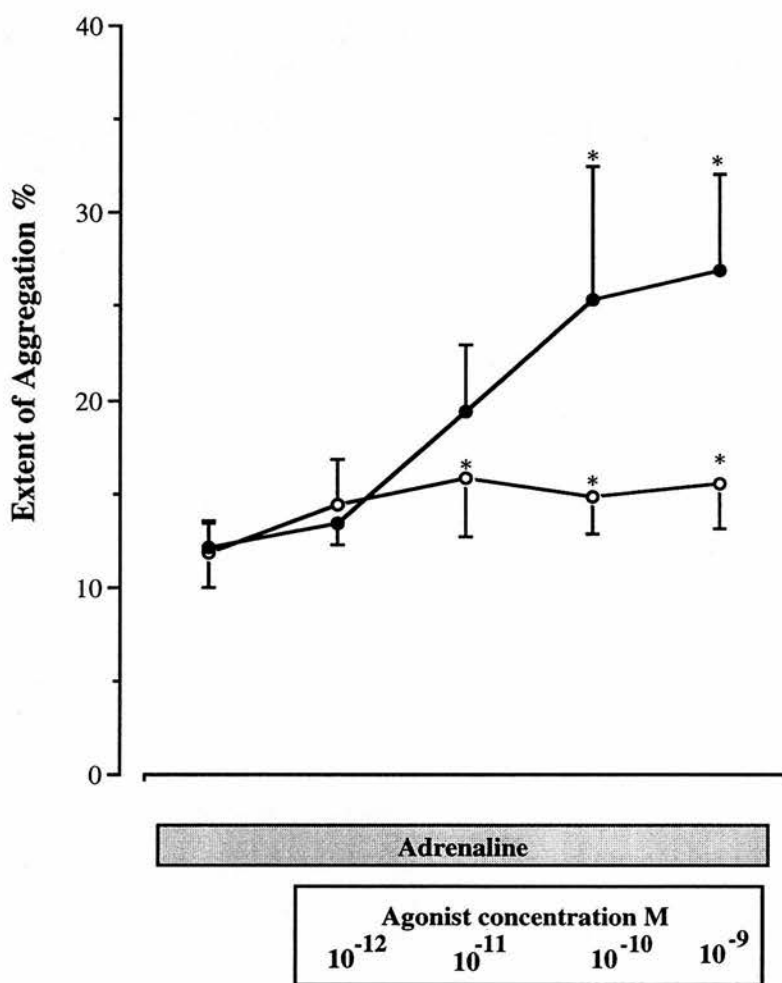


Figure 4.1. Potentiation of adrenaline-induced primary aggregation by ET-1 ○ ( $p=0.04$ ,  $n=12$ ) and SRTX S6c ● ( $p=0.05$ ,  $n=12$ ).



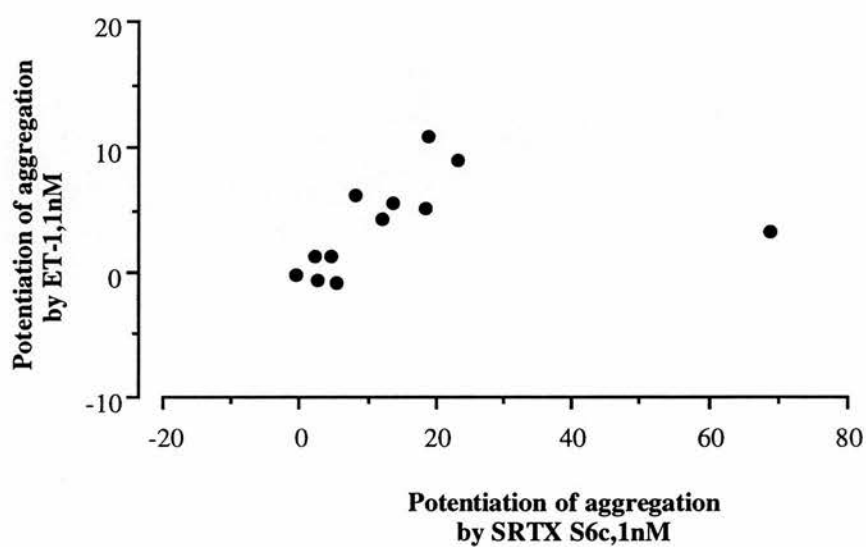


Figure 4.1b The increase, in percent aggregation, by ET-1, 1nM, plotted against the increase, in percent aggregation, by SRTX S6c, 1 nM in the same subjects, n=12.

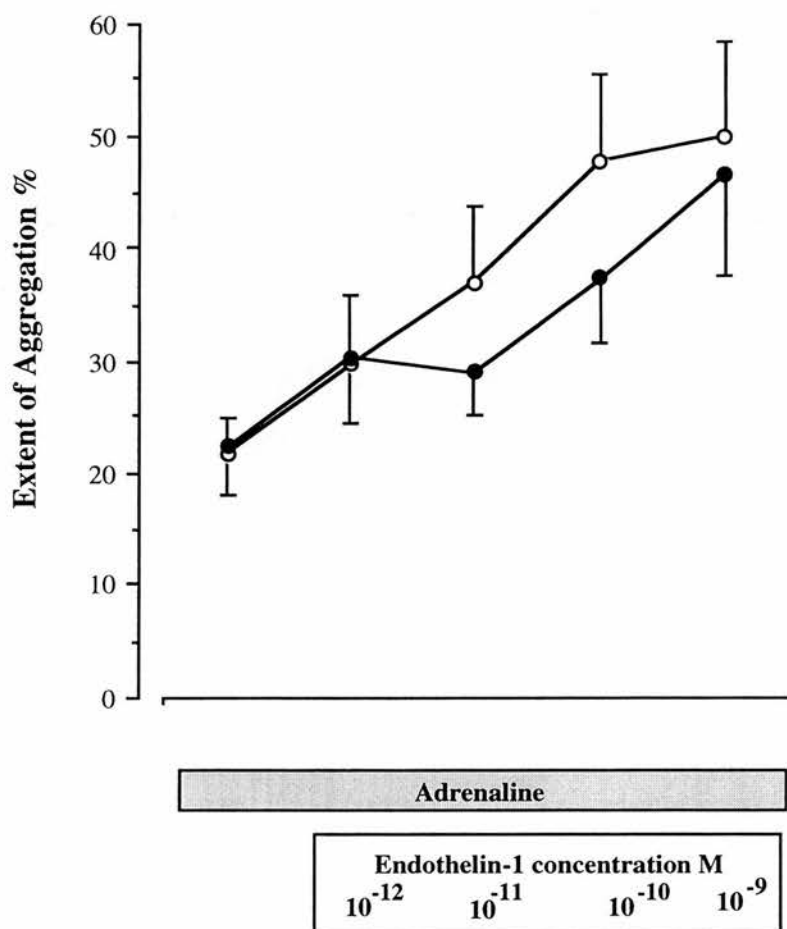


Figure 4.2 Reproducibility of ET-1 potentiation of adrenaline-induced primary aggregation in responders; ○ adrenaline + vehicle or ET-1, ● repeated dose response to ET-1 in platelet from the same donors, n=6.

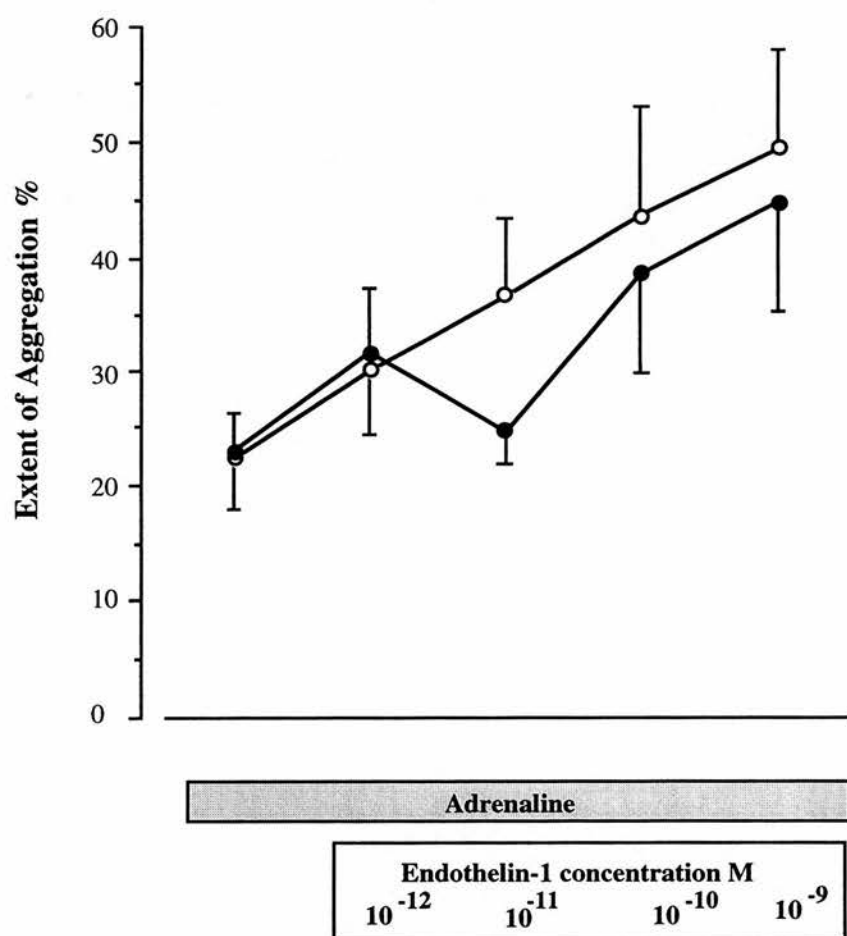


Figure 4.3. Platelet aggregatory response to adrenaline + ET-1 in the presence of vehicle ○ and BQ-123 ● (n=6).

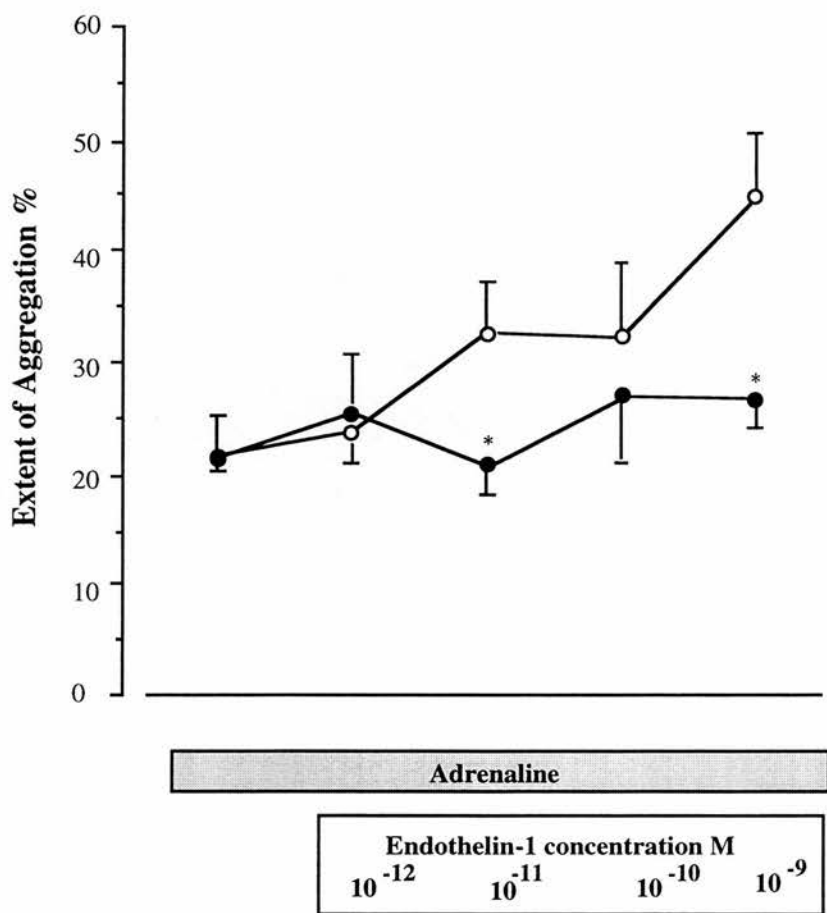


Figure 4.4 Platelet aggregatory response to adrenaline + ET-1 in the presence of vehicle ○ and BQ-788 ● (p = 0.03, n=6).

## **CHAPTER 5**

### **ENDOTHELIN RECEPTOR-MEDIATED ACCUMULATION OF PLATELET CYCLIC GMP**

## Introduction

As described in the preceding chapter ET-1 acts on target cells via cell surface ET<sub>A</sub> and ET<sub>B</sub> receptors. By binding to these receptors ET stimulates intracellular second messengers which in turn activate a functional change in the cell. Some physiological actions result directly from the intracellular messengers such as vascular smooth muscle cell contraction, while others, such as relaxation, can be induced indirectly through activation of an endothelial ET<sub>B</sub> receptor leading to release of the endothelium derived dilators prostacyclin and NO.

A number of intracellular signalling pathways have been associated with ET stimulation of cells; these include the phosphatidylinositol cycle (PI system), protein kinase C, cyclic AMP and cyclic GMP. Studies in preparations of isolated bovine vascular smooth muscle cells have shown that ET-1 increased cyclic AMP levels via the ET<sub>A</sub> receptor, presumably coupled to a G<sub>s</sub> protein, while in bovine endothelial cells the ET<sub>B</sub> receptor attenuated forskolin stimulation of adenylate cyclase (Eguchi, *et al.*, 1993). In transfected Chinese hamster ovary cells a similar pattern was observed (Aramori & Nakanishi, 1992). In rat aortic strip preparations, however, ET<sub>B</sub> receptor activation caused an increase in cyclic GMP which was abolished by pre-incubation with the NO synthase inhibitor N-monomethyl-L-arginine (Fujitani *et al.*, 1993). This suggests that ET<sub>B</sub> receptor activation could lead to an increase in NO production, which in turn increases cellular cyclic GMP levels, the second messenger for NO.

The work described in the previous chapters and published by others suggests that platelet aggregation is modulated in a bi-directional manner by ET receptor agonists. As described in chapter 1 platelets share many of the intracellular signalling mechanisms described above. The 'indirect' stimulation of guanylate cyclase

mediated by NO may also have a parallel in platelets due to presence of NO synthase in platelets. The modulation of platelet activity by both cyclic AMP and cyclic GMP has been extensively studied. To investigate the possible modulation of cyclic nucleotide second messengers in platelets by ET receptor activation the work presented in this chapter examined the effects of incubation of ET-1 and sarafotoxin S6c (SRTX S6c) *in vitro* on platelet cyclic AMP and cyclic GMP concentrations in human platelet rich plasma. The effect of *in vitro* incubation of platelet rich plasma with adrenaline on cyclic AMP and cyclic GMP has also been examined.



## METHODS

Healthy male subjects, aged 27 to 35 years, participated in this study.

PRP was prepared as for aggregation studies. PRP was divided into sub-aliquots and stored at room temperature under 95% O<sub>2</sub>:5% CO<sub>2</sub> until required. PRP was stirred at 37°C for 4 min prior to the addition of adrenaline ( $5 \times 10^{-7}$  -  $5 \times 10^{-5}$ ), ET-1 ( $10^{-11}$ – $10^{-7}$  M), SRTX S6c ( $10^{-11}$ - $10^{-7}$  M) or vehicle (0.9% saline). Dose-response studies to each agonist were performed in separate parallel aliquots. After 5 min, the platelet rich plasma was immediately centrifuged at 10 000 g for 2 min at 4°C, the plasma was removed, the platelet pellet resuspended in 2 ml ethanol and left at room temperature for 15 min. This suspension was centrifuged at 1200 g for 15 min and the supernatant decanted and dried down under air. The samples were resuspended in 500 µl assay buffer and stored at -20°C prior to assay.

Cyclic nucleotide (cyclic AMP and cyclic GMP) concentrations were measured using in-house radioimmunoassays described in chapter 2. Five hundred µl samples were acetylated; 50 µl of the samples were incubated for 18 hr at 4°C with 100 µl of antibody and 150 µl of [<sup>125</sup>I]-iodinated tracer. Antibody-bound tracer was separated from free tracer by centrifugation with activated charcoal at 4°C for 30 min at 1700 g. Free radioactivity in the charcoal was measured using an LKB multiwell gamma counter.

Cyclic nucleotide levels are expressed in pmol ml<sup>-1</sup> PRP.

## Statistics

Statistical analyses were by repeated measure ANOVA. For comparisons giving significant result by ANOVA the Fisher test was used to identify individual points that were significantly different from basal. For comparisons between separate dose response curves two way repeated measure ANOVA was used. Differences were considered to be statistically significant at the 5% level.

## RESULTS

Adrenaline caused a dose dependent increase in platelet cyclic AMP concentration ( $p = 0.004$ ) (fig. 5.1a) and an increase in platelet cyclic GMP concentration ( $p = 0.001$ ) (fig. 5.1b).

Neither ET-1 ( $p = 0.11$ ) nor SRTX S6c ( $p = 0.15$ ) caused a significant change in platelet cyclic AMP concentrations (fig 5.2). However, both ET-1 and SRTX S6c caused a significant dose-dependent increase in platelet cyclic GMP concentrations, ( $p < 0.05$  and  $p < 0.01$  respectively. fig 5.3.)

## DISCUSSION

Adrenaline, at a concentration of  $5 \times 10^{-5}$  M, increased platelet cyclic AMP and cyclic GMP concentrations. The effect of adrenaline on platelet cyclic AMP levels is not clearly understood. Co-incubation of adrenaline with  $\text{PGE}_1$  for 30 sec attenuated the prostaglandin stimulated increase in platelet cyclic AMP, interpreted by the authors as an adrenaline mediated inhibition of adenylate cyclase (Clare *et al.*, 1984). However, adrenaline increased platelet cyclic AMP concentrations in the presence of a phosphodiesterase inhibitor suggesting that adrenaline may also stimulate adenylate cyclase (Haslam & Taylor, 1971). Adrenaline can act on two

separate receptors on platelets, the  $\alpha_2$  receptor, which is linked to  $G_i$  protein which inhibits adenylate cyclase, and the  $\beta_2$  receptor, linked to a  $G_s$  protein which stimulates adenylate cyclase. It is possible that when platelet cyclic AMP is already elevated the effect of subsequent exposure to adrenaline is mediated by the  $G_i$  protein but in the absence of pre-elevated cyclic AMP levels the effect of adrenaline is mediated by the  $G_s$  protein. In the results reported in this thesis incubation of PRP with adrenaline for 5 min results in a significant increase in cyclic AMP, supporting the findings of Haslam & Taylor that adrenaline is able to stimulate adenylate cyclase in the absence of other adenylate cyclase activators. Cyclic GMP is known to be a second messenger for NO. The increase in cyclic GMP observed is consistent with adrenaline stimulating platelet NO, proposed in chapter 3 as a result of the potentiation of adrenaline-induced aggregation by the NO synthase inhibitor LNMMA. This could be further investigated by the use of a porphyrinic microsensor, as described by Malinski in the measurement of collagen induced platelet NO production (Malinski, *et al.*, 1993).

Incubation of platelet rich plasma with the non-selective  $ET_A/ET_B$  receptor agonist ET-1 or with the  $ET_B$  selective agonist SRTX S6c did not significantly alter platelet cyclic AMP levels. However, both agonists significantly increased platelet cyclic GMP. This would suggest that human platelets contain an ET receptor which is responsible for increasing platelet cyclic GMP concentrations either through stimulation of guanylate cyclase or by inhibition of a specific cyclic GMP phosphodiesterase. The precise mechanism could be explored by investigating the effect of an ET receptor agonist either in the presence of an inhibitor of soluble guanylate cyclase or maximal inhibition of phosphodiesterase activity. On the basis of the findings with SRTX S6c, this is most likely an  $ET_B$  receptor. SRTX S6c increased platelet cyclic GMP by up to approximately 80%. Bode-Böger demonstrated that L-arginine infusion caused a 45% increase in platelet cyclic GMP

which was associated with an inhibition of *ex vivo* aggregation to ADP of approximately 30% (Bode-Böger *et al.*, 1994). This would suggest that the SRTX S6c effect on cGMP observed here would be capable of causing a significant modulation of platelet aggregation. The marked elevation of cyclic GMP by SRTX S6c contrasts with the lesser response in cGMP seen with ET-1.

Although the difference in the cyclic GMP response to ET-1 and SRTX S6c was not statistically different, the disparity in the magnitude of the response was a curious and unexpected finding. ET-1 has previously been shown to inhibit sodium nitroprusside stimulated increase in cyclic GMP in human pulmonary vessels (Pussard *et al.*, 1995), and consequently it is possible that the difference in the effects of ET-1 and SRTX S6c observed here may reflect an ET<sub>A</sub> mediated inhibition of guanylate cyclase stimulation, or of an increase in cyclic GMP degradation. This could be investigated by comparing the effect of ET-1 in the presence and absence of the ET<sub>A</sub> antagonist BQ-123. There remain two other possibilities to explain the discrepancy. It has been reported that ET-1 is more sensitive to degradation by a neutral endopeptidase than SRTX S6c (Sokolovsky *et al.*, 1990). The receptor agonists were incubated in the plasma for 5 min, whereas the degradation times reported by Sokolovsky were hours rather than minutes, consequently it seems unlikely that this would have been an important factor. Nevertheless, it may be useful to measure the plasma ET and SRTX S6c levels over the incubation period to confirm this. The third possible explanation is based on the proposition that a receptor with the binding characteristics of the ET<sub>C</sub> receptor may be present in mammals. Although there is pharmacological evidence for such a receptor (Douglas, *et al.*, 1995) it has yet to be cloned from mammalian tissue. However, if such a receptor were to exist on human platelet it could be responsible for the apparent increased cyclic GMP accumulation evoked by SRTX S6c.

The mechanism responsible for the ET receptor mediated rise in cyclic GMP is not clear. Selective activation of the ET<sub>B</sub> receptor in rat aorta, in the presence of an intact endothelium, stimulates an increase in cyclic GMP and leads to relaxation of the aorta by a mechanism which can be blocked by the inhibition of NO synthase (Fujitani, *et al.*, 1993). This demonstrates that the ET<sub>B</sub> receptor is able to induce an increase in cyclic GMP in neighbouring vascular smooth muscle cells by stimulation of NO production in endothelial cells. In cultured epithelial cells both ET-1 and ET-3 were able to cause an increase in cyclic GMP in the target cell, by a mechanism which was also blocked by LNMMA (Ishii, *et al.*, 1991) demonstrating that ET<sub>B</sub> stimulated NO can have an autocrine as well as paracrine role.

The platelet L-arginine:NO pathway which was characterised in 1990, (Radomski, *et al.*, 1990) demonstrated that platelet derived NO could inhibit platelet aggregation through elevation of intra-platelet cyclic GMP. However, there is evidence for an insulin receptor linked mechanism for increasing platelet cyclic GMP which is independent of NO synthase, (Trovati *et al.*, 1994) and recently the benzylindazole derivative YC-1 has also been shown to inhibit platelet aggregation and activate soluble guanylate cyclase through a NO independent mechanism in human platelets (Wu *et al.*, 1995). This not only indicates that platelet guanylate cyclase may be activated by several different mechanisms but also confirms that the elevation of cyclic GMP *per se* inhibits platelet aggregation, probably by stimulation of cyclic GMP dependent protein kinases. To investigate the role of NO production, the ET-1/SRTX S6c stimulated rise in cyclic GMP studies could be performed in the presence of LNMMA.

In conclusion, the work presented in this chapter supports the proposition of an ET ET<sub>B</sub> receptor on human platelets. Activation of this receptor stimulates cyclic GMP

accumulation, which is known to be involved in the inhibition of platelet aggregation.



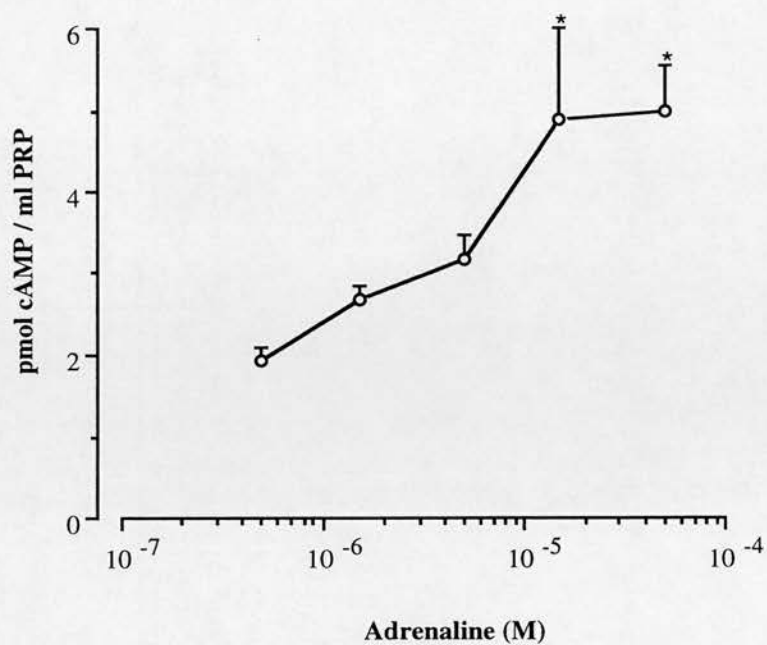


Figure 5.1a

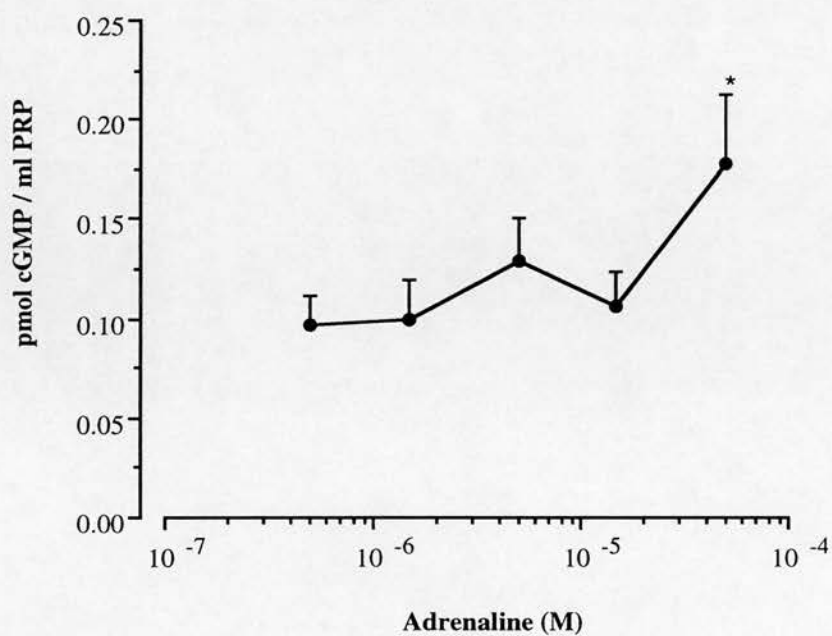


Figure 5.1b

Figure 5.1. Adrenaline induced accumulation of platelet cyclic nucleotides,  $n=6$ . Figure 5.1a, adrenaline induced an increase in platelet cyclic AMP, ( $\circ$ ),  $p=0.004$ , and figure 5.1b, platelet cyclic GMP, ( $\bullet$ ),  $p=0.001$ .



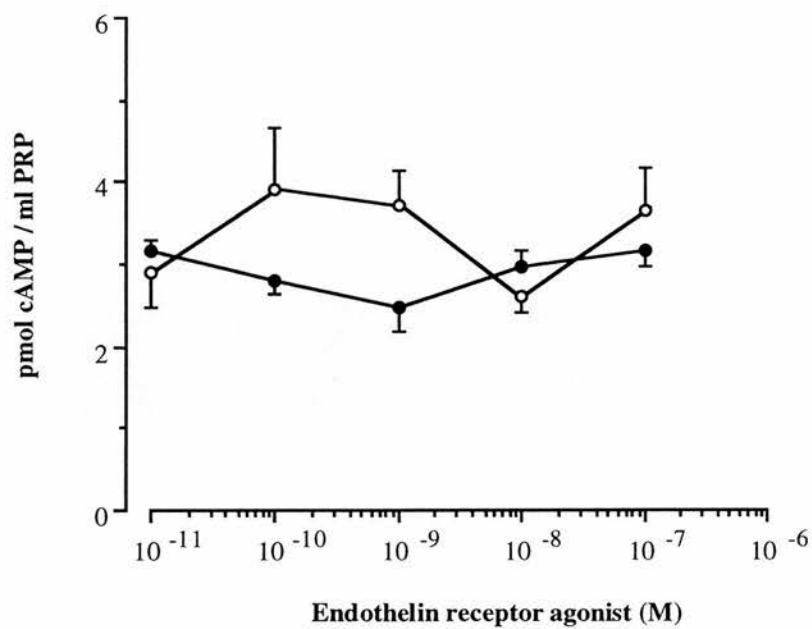


Figure 5.2 Effect on platelet cyclic AMP in response to treatment with ET-1 (○),  $p=0.11$  and SRTX S6c (●),  $p=0.15$ ,  $n=6$ .

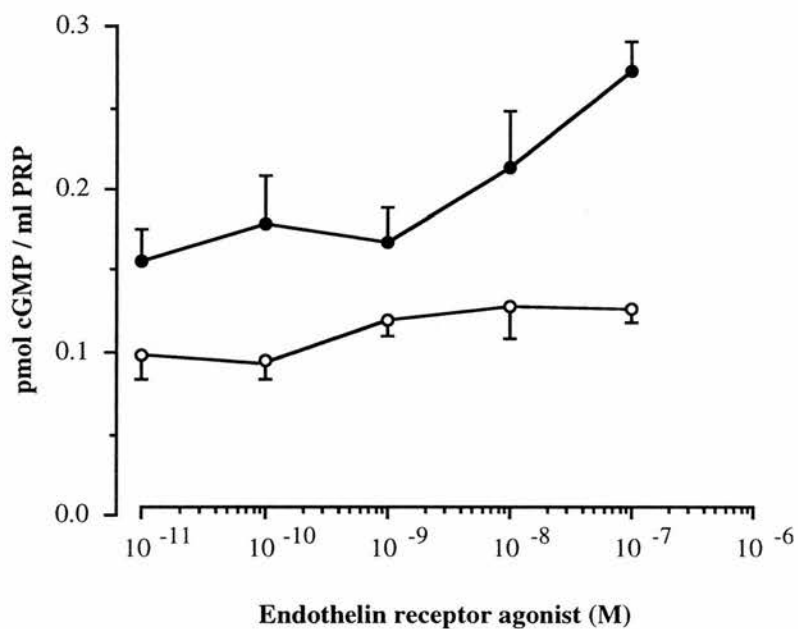


Figure 5.3. Change in platelet cyclic GMP in response to treatment with ET-1 (○),  $p=0.03$  and SRTX S6c (●),  $p=0.008$ ,  $n=6$ .

## **CHAPTER 6**

### **FURTHER INVESTIGATION INTO ENDOTHELIN RECEPTORS ON PLATELETS USING RECEPTOR BINDING STUDIES AND RT-PCR**

## INTRODUCTION

In the preceding chapters modulation of platelet aggregation by ET-1 has been described. In addition, evidence for activation of a second messenger system, platelet cyclic GMP, has also been given. ET-1 exerts effects on cells by binding to specific cell surface receptors, which in turn stimulate intracellular messengers. Other groups have also produced results for ET mediated responses in platelets indicating the involvement of specific ET receptors. These included changes in intracellular calcium and intracellular pH which, at least in the latter case, were inhibited by specific ET receptor antagonists (Astarie-Dequeker, *et al.*, 1992; Touyz & Schiffrin, 1993a). These findings provide strong evidence for the existence of ET receptors on platelet membranes. However, strictly speaking they only describe an association of a stimulus and an effect which is consistent with the existence of cell surface ET receptors.

To date, however, no ET receptor has been conclusively identified on platelets. Ligand binding experiments provide more direct evidence for the existence of a receptor. A dissociation constant ( $K_D$ ), derived from competition binding studies with a fixed concentration of radio-labelled agonist and increasing concentrations of unlabelled agonist, can be used to describe the equilibrium between receptor-bound and unbound ligand.

Molecular biology provides an alternative method for indicating the existence of a specific receptor. As described in chapter 1, platelets do not possess nuclei. They are formed from the fragmentation of the cytoplasm of nucleated megakaryocytes. Platelets do not, therefore, contain DNA and, consequently lack the template for the synthesis of mRNA. However, in 1989 Roth and colleagues detected mRNA for the glycoproteins 1b $\alpha$  and 1b $\beta$ , in platelet preparations from patients with essential

thrombocythaemia, but not in preparations of other blood cells (Roth *et al.*, 1989). They concluded that vestigial amounts of platelet parent megakaryocyte mRNA remained detectable in circulating 'young' platelets. More recently, platelet angiotensin receptor mRNA was quantified, and found to correlate inversely with plasma angiotensin II levels (Shibata, *et al.*, 1994).

In 1992, Diochot showed that the MEG 01 cell line, a model for megakaryocyte cells that expresses the glycoprotein GP IIb, responds to ET-1, suggesting that megakaryocytes have, and therefore platelets might have, ET receptors (Diochot *et al.*, 1992). Recently the same group isolated ET<sub>B</sub> receptor mRNA from MEG 01 cells (Hamroun *et al.*, 1995).

It was the aim of the work described in this chapter to identify specific ET binding sites by using ligand binding experiments which would indicate the presence of an ET receptor on platelet membranes. Before performing competition binding curves, preliminary studies were carried out to determine optimal assay conditions. Two sets of controls were performed. The first to confirm the validity of the methods used for identifying ET-1 receptor binding and the second to confirm that receptor binding sites on platelets could be identified. Competition binding studies were performed in rat cardiac membranes, a tissue-type known to express ET receptors, and on platelet membranes using the adrenergic  $\alpha_2$  receptor antagonist yohimbine. To further strengthen the evidence for a specific receptor and to link the existence of the proposed binding with an ET receptor gene a second study was devised with the aim of isolating platelet mRNA and identifying ET receptor message in platelets.

## METHODS

### Ligand binding study

#### *Optimisation of assay conditions*

##### Optimisation of incubation time

Three aliquots of platelet membranes, 0.2 mg of protein /tube, prepared as described in chapter 2, were incubated with [ $^{125}$ I]-ET-1 (0.5 nM,  $\approx$  100 000 cpm) and either buffer or ET-1 ( $10^{-6}$  M) to determine total and non specific binding respectively. Incubations were performed at 37°C for 0, 15, 30, 60, 90 & 120 min. The reaction was stopped by the addition of ice cold buffer, bound and free [ $^{125}$ I]-ET-1 were separated by rapid filtration, and the bound fraction was quantified by counting the filters for three min in a gamma counter. Experiments were performed in duplicate.

##### Optimisation of protein concentration

Three aliquots of platelet membranes were incubated with [ $^{125}$ I]-ET-1 (0.5 nM  $\approx$  100 000 cpm) and either buffer or ET-1 ( $10^{-6}$  M) at 37°C for 60 min, to determine total and non specific binding respectively. Incubations of platelet membranes were performed using protein concentrations of 0.03, 0.06, 0.12, 0.25 and 0.5 mg ml $^{-1}$ . The reaction was stopped, by the addition of ice-cold buffer followed by rapid filtration, and the bound radioactive fraction was prepared and radioactivity measured as described above.

### *Competition curves*

Competition binding curves were performed for ET-1 using rat cardiac and platelet membranes and for yohimbine using platelet membranes. Precise assay conditions are given in chapter 2. Briefly, rat cardiac membranes were incubated at 37°C at a final concentration of 0.5 mg protein/tube and platelets at 0.4 mg /ml, [<sup>125</sup>I]-ET-1 was used at a final concentration of 0.5 nM ( $\approx$  100 000 cpm) and incubation was for 60 min, [<sup>3</sup>H]-yohimbine was used at a final concentration of 2 nM ( $\approx$  20 000 cpm). All binding studies were performed at 37°C. Binding curves with a fixed concentration of radio-labelled agonist and increasing concentrations of unlabelled agonist were performed and dissociation constants ( $K_D$ ) were determined using the computer programme Ligand (Munson & Rodbard, 1980).

### *Protein concentration*

All protein concentrations were determined by the Bio-Rad protein assay based on the Bradford dye binding procedure. Protein standards were prepared containing 0, 0.1, 0.2, 0.4, 0.8, 1.0 mg protein/ml of albumin. A 40  $\mu$ l aliquot of standard or sample was incubated for 10 min at room temperature, after which optical density was measured, and protein concentration calculated.

Statistical analysis was by repeated measures ANOVA.

## **RT-PCR study**

### *Platelet preparation*

Platelets were prepared by two separate methods.

### *Method 1*

The first method was an adaptation of the method described by Shibata (Shibata, *et al.*, 1994). Plasma was prepared from venous blood by centrifugation. The plasma was then diluted in 20 vol. Medium 199 and centrifuged for 10 min at 1000 *g*. The pellet was resuspended in the above medium and both supernatant and resuspended pellet were centrifuged at 1000 *g* for 20 min. Both subsequent pellets were frozen in liquid nitrogen and identified as platelet poor and platelet rich pellets respectively.

The platelet rich pellet resulted in approximately 10 fold greater harvest of platelets compared to the platelet poor pellet. Both preparations had a contamination of less than 1:1000 white blood cells, however white cells were only detected in 2 of 6 platelet poor samples.

### *Method 2*

The second protocol for platelet preparations involved purification of platelets on discontinuous gradients of percoll as described in chapter 2. No whole cells other than platelets were detected in samples prepared by this method.

### *RT-PCR Protocol*

Extraction of RNA was performed using chloroform as described in chapter 2. RNA was also extracted from MEG 01 cells to act as a positive control.

RT-PCR was performed using selective oligonucleotide primers for ET<sub>A</sub> and ET<sub>B</sub> receptors, described in chapter 2. Samples prepared by method 2 were only tested using primers for the ET<sub>B</sub> receptor. MEG 01 RNA and a blank sample containing water in place of RNA were analysed as positive and negative controls respectively.



Elution of the RT-PCR product on the agarose gel was always performed in the presence of molecular weight markers. A result was considered positive if a single band was visible at the expected position compared to the molecular weight marker and the positive control. The predicted sizes for the amplified receptor cDNA products were 368 base pairs for the ET<sub>A</sub> receptor and 238 base pairs for the ET<sub>B</sub> receptor.

## RESULTS

### Ligand binding study

#### *Incubation time*

Specific binding, calculated by the subtraction of non-specific binding, that is binding in the presence of ET-1 ( $10^{-6}$  M), from total binding appeared to peak at 60 min. However, specific binding did not change significantly as a function of time ( $p=0.11$ ,  $n=3$ ) (fig 6.1).

#### *Protein concentration*

There was a linear relationship between specific binding and protein concentration over the concentrations examined (fig. 6.2). The relationship between non-specific binding and protein concentration was linear up to approximately  $0.5 \text{ mg ml}^{-1}$ . In subsequent competition binding curves protein concentrations of  $0.4 \text{ mg ml}^{-1}$  were used.

#### *Competition Binding Curves*

Unlabelled ET-1 dose dependently reduced the binding of [ $^{125}\text{I}$ ]-ET-1 to rat cardiac membrane preparations ( $p=0.0001$ ;  $n=6$ ) in a manner similar to that previously reported (Waugh, *et al.*, 1992). The mean  $K_D$  for 6 experiments was  $0.85 \pm 0.52 \text{ nM}$  (Fig 6.3).

Unlabelled yohimbine dose dependently reduced the binding of [ $^3\text{H}$ ]-yohimbine in platelet membrane preparations ( $p=0.0001$ ;  $n=3$ ), the mean  $K_D$  for 3 experiments was  $1.28 \pm 0.60 \text{ nM}$  (Fig 6.4).

In platelet membranes, unlabelled ET-1 did not consistently influence [ $^{125}$ I]-ET-1 binding ( $p=0.18$ ;  $n=6$ ) (Fig 6.5). The larger standard errors were due to an apparent dose dependent reduction in binding in platelet preparations from two subjects only, while the other four subjects appeared to show only non-specific binding. For the two subjects who appeared to show reduced binding with increasing concentrations of cold ligand data analysis produced  $K_D$ s of 1.1 and 0.4 nM.

### **RT-PCR study**

#### *Extraction method 1.*

The platelet rich pellet obtained using this extraction method was positive for mRNA for both the  $ET_A$  and the  $ET_B$  receptors, however the platelet poor pellet was only positive for  $ET_B$  receptor mRNA (fig 6.6). MEG 01 cells were positive for both receptor types.

#### *Extraction method 2.*

In the first run of samples prepared by the use of percoll,  $n=3$ , all samples were positive for  $ET_B$  receptor mRNA. However, the blank sample containing water in place of RNA appeared to exhibit a faint band, indicating slight cross contamination (fig 6.7). In the second run only 1 of the 3 samples analysed produced a positive result.

### **DISCUSSION**

Dissociation constants obtained for the competition binding studies of ET-1 on rat cardiac membranes and for yohimbine on platelet membranes,  $0.85 \pm 0.52$  nM and

1.3  $\pm$  0.6 nM respectively were consistent with previous findings (Brown *et al.*, 1990; Gu, *et al.*, 1989). This suggests that the experimental procedures employed in the ligand binding study were valid. The preliminary studies which examined varying incubation time and protein concentration showed a small, but in the latter case significant specific binding of [<sup>125</sup>I]-ET-1 to platelet membranes. However, there was not a consistent reduction of binding with incubation of increasing concentrations of ET-1 in all subjects. Nevertheless, two subjects did display apparent competitive binding curves with KDs of similar order of magnitude to that observed in the cardiac membranes.

The competition binding studies with yohimbine were successful in producing results consistent with the findings of other groups, confirming the presence of the  $\alpha_2$  receptor. However, the mean number of  $\alpha_2$  receptors is between 200 and 300 per platelet (Blockmans, *et al.*, 1995). The potentiating effect of ET-1 on platelet aggregation is similar to that of angiotensin II, and human platelets have only 4 to 6 receptor sites/platelet for angiotensin II (Ding *et al.*, 1984). It is possible therefore, that when receptor numbers are low, as seem likely for ET-1, this method of membrane preparation and analysis may be insensitive in their detection. A possible option to overcome low receptor number might be to increase the protein concentration, and hence the amount of platelet membrane, in the assay. However, experience suggests that this would result in greater non-specific binding. Another possibility is that detection of binding sites is hampered by a rapid down-regulation of the platelet receptors. Consequently, it may be possible to identify the receptors by altering assay conditions, such as performing the incubation at low temperatures. This may introduce other possible complications such as a long incubation time, where the integrity of the membranes and the stability of the agonists would have to be verified.

An alternative to using platelet membrane preparations would be to use whole platelets. Studies of angiotensin II binding to human platelets have tended to use whole platelets (Ding, *et al.*, 1984; Mann *et al.*, 1985). The use of whole platelet may reduce non-specific binding of the ligands and may therefore give greater 'signal to noise ratio' enhancing the sensitivity of detection. Difficulties in using whole platelets involve receptor internalisation and the inability to freeze and defrost intact platelets.

In the second study looking at receptor mRNA, the results obtained from samples prepared by the method described by Shibata (1994) indicated that platelet preparations contain mRNA for the ET<sub>A</sub> and the ET<sub>B</sub> receptors. However, although a contamination by other blood cells of 1:1000 may not be important in functional studies, RT-PCR is a highly sensitive procedure involving exponential amplification of the reverse transcriptase product. Nucleated cells, such as neutrophils, would be expected to contain much more RNA than platelets, and consequently contamination by such cells might affect the results obtained by RT-PCR. The platelet poor samples, which contained less contamination may be a better indicator of platelet mRNA. The results from these samples indicate that platelets contain only ET<sub>B</sub> receptor mRNA. However, these samples were not completely free of contamination. The second platelet preparation protocol produced samples with no evidence of contamination by other cells. Unfortunately, although these results suggest the presence of ET<sub>B</sub> mRNA, analysis of these samples produced inconsistent results, with samples from some subjects producing positive results for the presence of ET receptors and others producing negative results.

One possible explanation for the results obtained by using method 1 is that platelets may contain ET<sub>B</sub> mRNA and that other blood-borne cells contain ET<sub>A</sub> mRNA. It is known that ET-1 acts on other circulating cells apart from platelets, including

monocytes and neutrophils (Elferink & Dekoster, 1994; Heslet *et al.*, 1993). Although the receptor subtype on monocytes has not yet been identified, the effects of ET on neutrophils are thought to be mediated by the ET<sub>A</sub> receptor (Elferink & Dekoster, 1994). It is, therefore, possible that the presence of ET<sub>A</sub> mRNA in the platelet rich sample can be accounted for by neutrophil contamination.

The fact that in the ligand binding study platelets from two volunteers appeared to display competition binding curves might suggest that part of the problem may be due to the fact that not all individuals express platelet ET receptors or that some have higher numbers of receptors. This would be consistent with the suggestion by Matsumoto of responders and non-responders to ET (Matsumoto, *et al.*, 1990) and the rather variable responses reported in chapters 3 and 4 (see figures 3.5 and 4.1b). This would also explain why receptor mRNA was not detectable in all the samples in the second study in this chapter. (It is important to note that two separate groups of volunteers were used in the two studies in this chapter). This hypothesis could be explored by screening volunteers for responders and non-responders using functional studies, and subsequently investigating receptor binding in the two groups.

With regards to the RT-PCR study it is important to be aware of possible limitations in the methods employed for purifying the platelet preparations. Platelets were separated from other blood cells on the basis of density, using centrifugation in method 1, and using centrifugation in percoll in method 2. These methods were used to obtain purified platelet preparations. However, by choosing the purest fraction, platelets of a greater density may be excluded. It has been suggested that greater platelet density and volume, features that are determined by the size of the parent megakaryocyte, are associated with increased platelet activity (Martin *et al.*, 1983). For this to be true it is likely that influences on platelet activity, such as receptor type, receptor number, or platelet biochemistry differ with platelet size. Therefore,

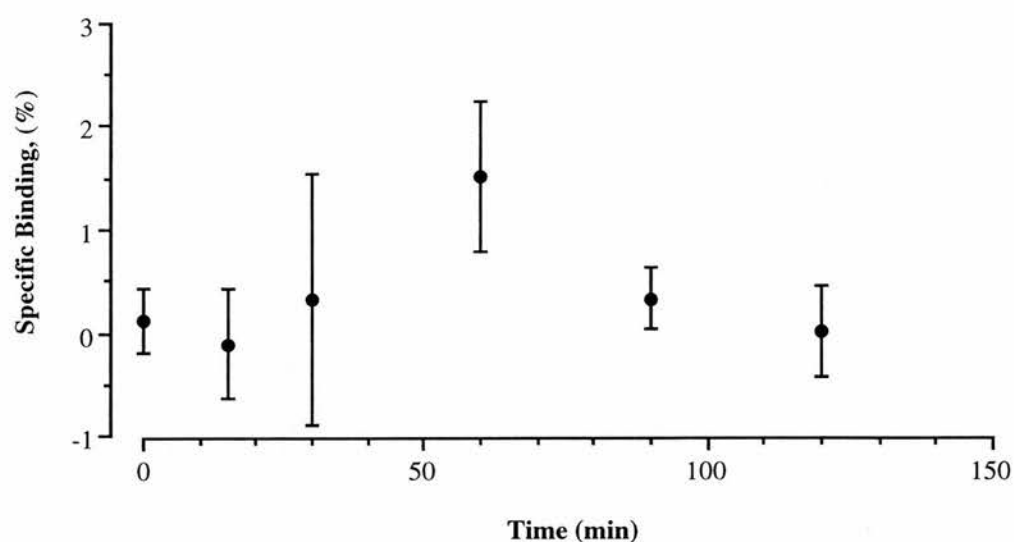


although the platelet rich fraction obtained using method 1 may have contamination by other blood cells, it also may contain a broader spectrum of platelet densities. (These problems would also apply when obtaining whole platelets for ligand binding studies.) It would, then, be incorrect to assume that the presence of ET<sub>A</sub> receptor mRNA is solely due to contamination from other blood borne cells. It may, therefore reflect ET<sub>A</sub> receptors being present on larger, denser platelets. There are at least two ways this problem might be addressed.

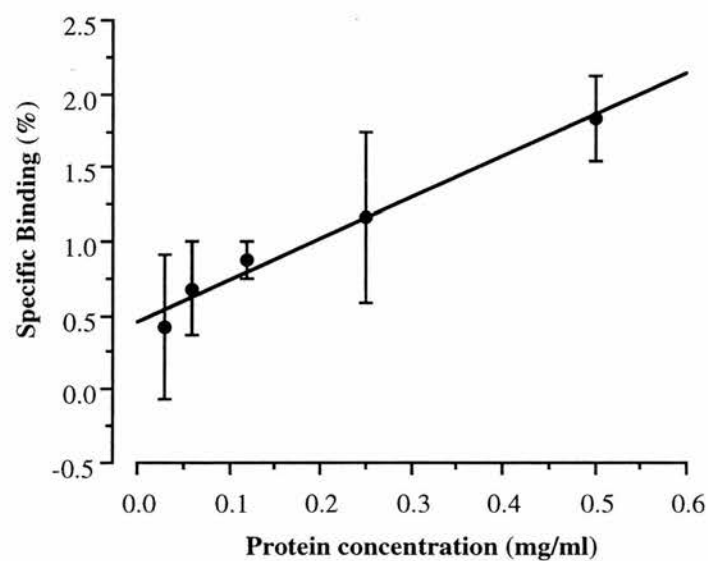
If a size distribution for the platelets were obtained from the whole blood sample and the resulting platelet sample, the two could be compared. This would allow confirmation that the platelet sample analysed represented the same distribution of platelets present in the blood. Alternatively, platelets could be collected and purified on the basis of a criterion other than density. For example, platelets could be isolated using an antibody raised against a platelet specific antigen, such as the GP IIb/IIIa complex. Although this method of preparation may produce the best results it would require some validation and would have to be practicable, for dealing with relatively large volumes of blood.

In conclusion the results of the studies reported in this chapter suggest that, firstly platelets from some, but not all, individuals possess receptors for ET-1 and secondly that preparations of blood-borne cells contain mRNA for both the ET<sub>A</sub> and the ET<sub>B</sub> receptor, and that platelets, from at least some individuals, contain mRNA for the ET<sub>B</sub> receptor.

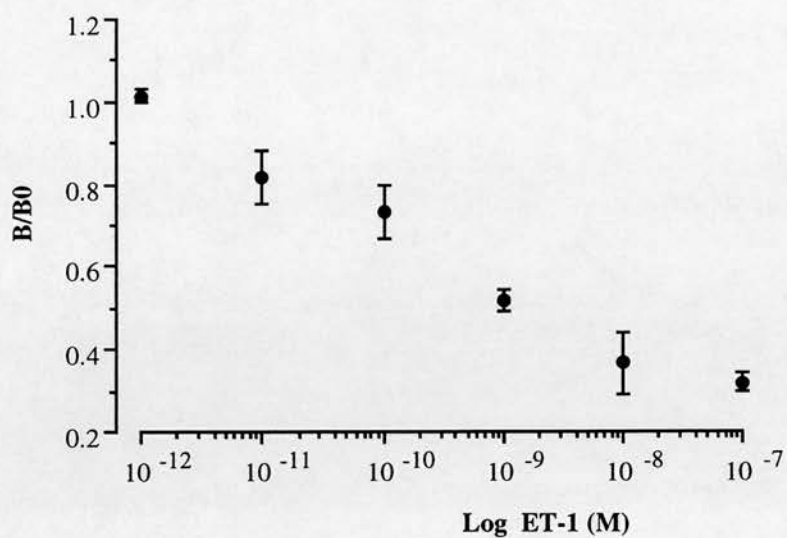




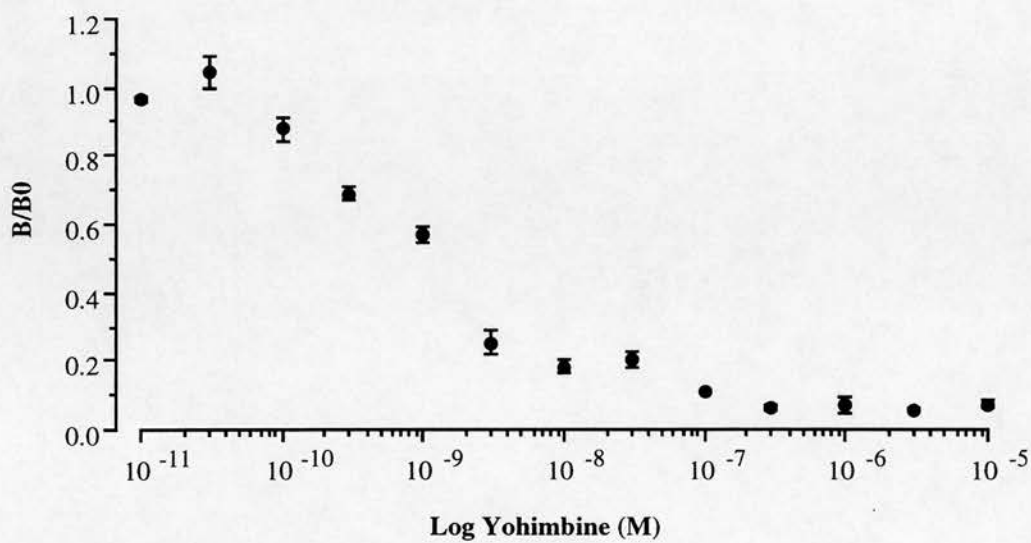
**Figure 6.1** Specific binding of [ $^{125}$ I]-ET-1 to platelet membranes as a function of time. Despite an apparent peak in specific binding as a percentage of total radioactivity, the differences are not statistically significant ( $p=0.11$ ,  $n=3$ ).



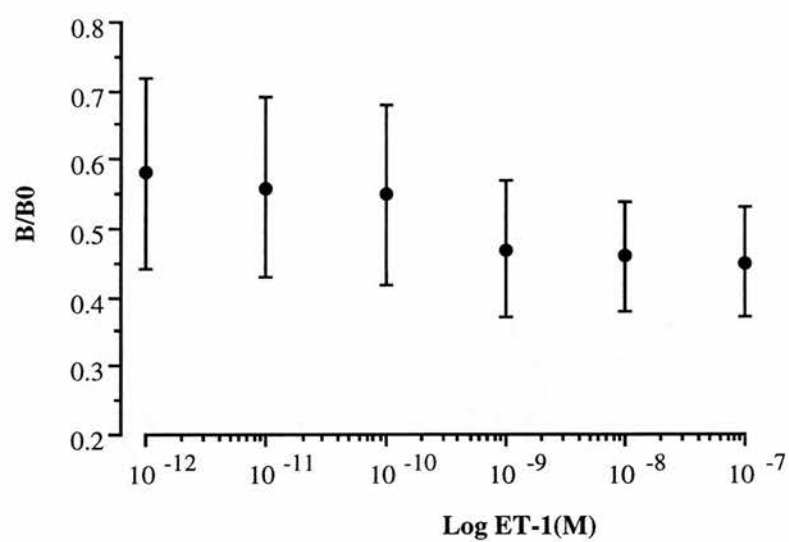
**Figure 6.2** Specific binding of [ $^{125}$ I]-ET-1 to platelet membranes as a function of protein concentration.



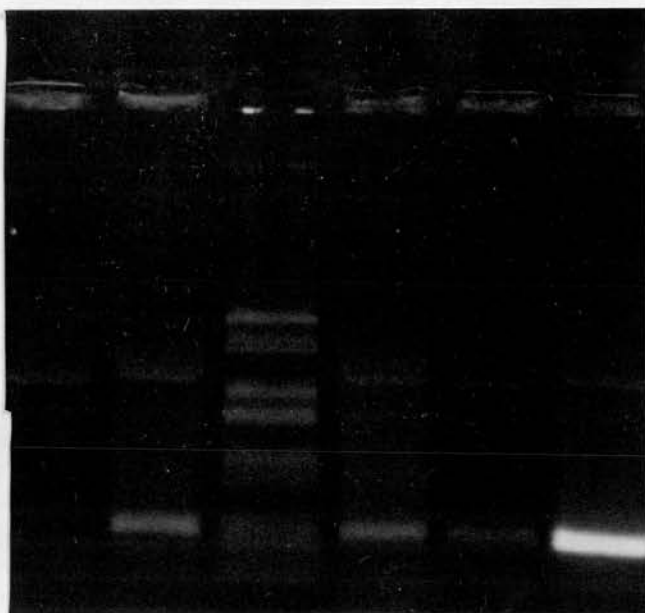
**Figure 6.3** [ $^{125}\text{I}$ ]-ET-1 binding to rat cardiac membranes in the presence of cold ET-1. (mean  $K_D$   $0.85 \pm 0.52$  nM,  $n=6$ )



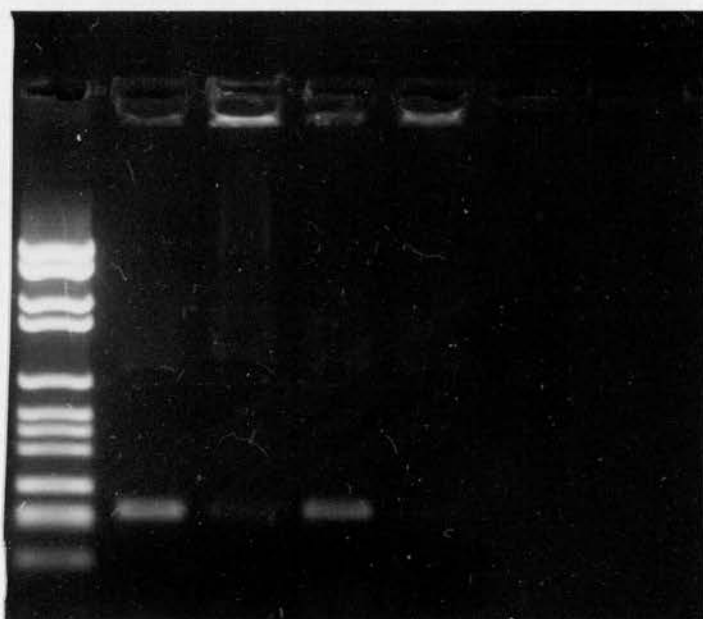
**Figure 6.4** [ $^3\text{H}$ ] yohimbine binding to human platelet membranes in the presence of yohimbine. (mean  $K_D$   $1.28 \pm 0.6$  nM;  $n=3$ )



**Figure 6.5** [ $^{125}\text{I}$ ]-ET-1 binding to human platelet membranes in the presence of ET-1 (n=6).



**Figure 6.6** A photograph of a gel, after loading with 3 'platelet poor' (pp) samples, showing bands of 238 base pairs, consistent with the presence of ET<sub>B</sub> mRNA. The gel was loaded as follows; lane 1 blank, lane 2 sample pp1, lane 3 molecular weight markers, lane 4 pp2, lane 5 pp3 and lane 6 MEG 01.



**Figure 6.7** A photograph of a gel, after loading with 3 samples prepared as described in method 2, showing bands of 238 base pairs, consistent with the presence of ET<sub>B</sub> mRNA, including a faint band in lane 5. The gel was loaded as follows; lane 1 molecular weight, lane 2 sample 2.1, lane 3 sample 2.2, lane 4 sample 2.3 and lane 5 blank.

## **CHAPTER 7**

### **INVESTIGATION INTO THE EFFECTS OF ENDOTHELIN-1, ADMINISTERED IN VIVO, ON EX VIVO HUMAN PLATELET AGGREGATION**

## INTRODUCTION

In chapters 3 and 4 of this thesis the effect of ET-1 on aggregation of human platelets *in vitro* was studied. Evidence was presented for a biphasic effect on aggregation, a potentiation of primary aggregation and an inhibition of secondary aggregation. Several other studies have been published, including some of the earliest studies on the effects of ETs on platelets, examining the *in vivo* and *ex vivo* effect of ET-1 and ET-3 on platelet aggregation. These studies used systemic doses of peptide in animal models and consistently reported an inhibition of aggregation in the dog and the rabbit (Hermán, *et al.*, 1989; Lidbury, *et al.*, 1989; Thiernemann, *et al.*, 1989). Several of these studies concluded that the effect of ETs observed was largely or wholly the result of ET<sub>B</sub> mediated prostacyclin release (Hermán, *et al.*, 1989; Hermán *et al.*, 1993; Thiernemann, *et al.*, 1989). No pro-aggregatory effect was reported.

Although prostacyclin mediated increase in platelet cyclic AMP and the subsequent inhibition of aggregation may be an important regulatory mechanism of platelet activity *in vivo*, platelets do not produce prostacyclin. Hence, this mechanism can not be responsible for the inhibitory action of ETs seen *in vitro* reported in earlier chapters and by others (Astarie-Dequeker, *et al.*, 1992; Touyz & Schiffrin, 1995). Although it is possible that the powerful inhibition of platelet aggregation by prostacyclin might have masked a potentiation of aggregation, one might expect this effect to have been revealed when prostacyclin synthesis was inhibited, but no such effect was reported (Thiernemann, *et al.*, 1989). Consequently, it would appear that the effects of ET-1 reported in these animal studies are not completely consistent with the effect observed in *in vitro* human platelet studies.

ET mediated responses are known to display interspecies variation in the vasculature (Reynolds *et al.*, 1995) and in platelets (Ohlstein, *et al.*, 1990). Consequently, it is not necessarily appropriate to extrapolate from previous studies in animals to effects of ET-1 in humans. In addition, the ETs are thought to be locally acting hormones. In the animal studies mentioned above systemic doses of ET were used and local actions of ET-1 may be obscured by the effects of systemic administration on the heart and kidney and the consequent change in blood flow and hormone production. The actions reported may not necessarily be consistent with a paracrine role of ET-1.

The aim of the work presented in this chapter was to investigate the effect of locally acting concentrations of ET-1 on *ex vivo* platelet aggregation in man. By using a dose of ET-1 known to act selectively to decrease blood flow in the infused forearm (Clarke *et al.*, 1989; Haynes & Webb, 1994) we have an effective bioassay of ET-1 efficacy *in vivo*. To assess the effects of prostanoid generation on the actions of ET-1, *ex vivo* studies were carried out in the presence and absence of cyclooxygenase inhibition by aspirin. At the dose used the local concentrations of ET-1 achieved are lower than those previously used for *in vitro* platelet aggregation studies. Consequently, we also examined the *in vitro* effect of ET-1 at a low concentration in an environment free of endothelium derived mediators.



## METHODS

### Subjects

Healthy male subjects, aged 20 - 36 years participated in these studies. No subject received vasoactive or non-steroidal anti-inflammatory drugs in the week before each study or alcohol or caffeine on the day of the studies. Clinical studies were carried out, with subjects recumbent, in a quiet laboratory maintained at a constant temperature of 24-26°C.

### Techniques

#### *Forearm Blood Flow*

Forearm blood flow measurements are given in detail in chapter 2, therefore only an outline is given below.

The left brachial artery was cannulated under local anaesthesia (1% lignocaine). Saline (0.9%) and ET-1 (5 pmol ml<sup>-1</sup>) were infused at a rate of 1 ml min<sup>-1</sup>. Blood flow was measured in both arms by venous occlusion plethysmography. The hands were excluded from the circulation during each measurement period. The percentage change in blood flow was calculated as percentage change from basal in the ratio of blood flow between the infused and non-infused arms.

#### *Platelet aggregation*

Platelet aggregation studies were carried out as described in previous chapters.

Blood for Ir-ET-1 assay was taken into EDTA and measured using a commercial radioimmunoassay kit as described in chapter 2.

### **Study design**

The study consisted of three phases, two *ex vivo* phases ( $\pm$  aspirin) and an *in vitro* phase. There was a minimum interval of seven days between phases and the order of phases was randomised for each volunteer. In the two *ex vivo* phases subjects received brachial artery infusions of saline (0.9%) for 30 min followed by ET-1, at 5 pmol min<sup>-1</sup>, for 60 min. Forearm blood flow was measured before, and 10, 20, 40 and 60 min after the start of the ET-1 infusion. Blood was sampled at +60 min from indwelling cannulae in both infused and control arms for *ex vivo* platelet aggregation and plasma ET concentrations. In platelet aggregation studies, platelets were challenged *in vitro* with ADP or adrenaline; both at concentrations of 1, 10 and 100  $\mu$ M. Dose response studies to ADP and adrenaline were performed in parallel. In the phase of the *ex vivo* study involving aspirin administration, aspirin 600 mg, was given intravenously 30 min before and 30 min after the start of ET-1 infusion.

In a third phase blood was sampled via a 19 g needle from subjects who had rested supine for 20-30 min. Platelets were incubated with either vehicle or ET-1, 100 pM for 1 min prior to the addition of vehicle, ADP or adrenaline at the same concentrations as described above.

## Statistical Analysis

Results are expressed as mean  $\pm$  s.e.mean. Statistical analysis was by repeated ANOVA for forearm blood flow studies. Student's *t*-test was used for comparisons of primary and secondary platelet aggregation and the plasma ET-1 comparison, with and without ET-1 administration.

## RESULTS

### Ex vivo

Brachial artery infusion of ET-1 caused significant forearm vasoconstriction, with a  $32.8\% \pm 5.3$  reduction in blood flow at 60 min ( $p < 0.001$ ). Pre-treatment with intravenous aspirin 600 mg significantly increased the forearm vasoconstriction, with a  $50.3\% \pm 5.9$  reduction in blood flow at 60 min ( $p < 0.001$  vs basal;  $p < 0.05$  ET-1 alone) (Fig 8.1).

Plasma ET-1 levels were significantly higher in venous blood collected from the infused arm compared to the control after 60 min, control arm vs infused arm, ( $2.5 \pm 0.7$  vs  $15.1 \pm 1.3$  pM,  $p < 0.001$ ).

ET-1 infusion did not significantly alter *ex vivo* adrenaline or ADP induced primary aggregation or ADP induced secondary aggregation ( $n = 12$ ). However, adrenaline induced secondary aggregation was significantly attenuated in plasma from the infused arm compared to the control ( $38.5 \pm 8.7$  vs  $67.1 \pm 6.6$ ,  $p < 0.05$ ,  $n = 8$ ) (Fig 8.2). Secondary aggregation to adrenaline was only seen in 8 of the 12 subjects.

Aspirin infusion did not significantly affect basal blood flows, blood pressure, heart rate or basal platelet aggregation (Table 7.1).

In aspirin treated volunteers, there remained no significant difference in the extent of primary aggregation to ADP in plasma from the non-infused ( $16.9 \pm 3.3$ ) compared to the ET-1 infused arm ( $12.4 \pm 3.3$ ). Similarly, secondary aggregation to ADP was not altered by ET-1 infusion in the presence of systemic aspirin ( $62.7 \pm 4.0$  vs  $61.2 \pm 5.9$  control arm vs ET-1 infused arm). No difference was seen in adrenaline induced

primary aggregation in plasma from the control arm compared to the ET-1 infused arm ( $13.8 \pm 1.7$  vs  $14.6 \pm 2.2$ ). Only 3/12 aspirin treated volunteers demonstrated adrenaline induced secondary aggregation with  $54.3 \pm 10.0\%$  aggregation in plasma from the control arm and  $47.5 \pm 14.7 \%$  in plasma from the infused arm.

### *In vitro*

Adrenaline and ADP induced primary aggregation tended to be increased by pre-incubation with ET-1, 100 pM, *in vitro* but this was not statistically significant, (Fig 3). However, secondary aggregation to adrenaline in the presence of ET-1 ( $39.7 \pm 8.7 \%$ ) was significantly attenuated compared to control ( $76.8 \pm 6.0 \%$ ;  $p < 0.01$ ,  $n = 8$ ). Secondary aggregation to ADP was not significantly altered by pre-incubation with ET-1 (Fig 8.3).

## Discussion

This study confirms that ET-1 causes sustained forearm vasoconstriction (Clarke, *et al.*, 1989; Haynes & Webb, 1994) and demonstrates for the first time that inhibition of prostanoid production potentiates ET-1 induced constriction in forearm resistance vessels. ET-1 did not significantly potentiate primary aggregation to ADP or adrenaline in any of the three phases of the study. However, both *ex vivo* and *in vitro* secondary aggregation to adrenaline was inhibited by pre-incubation with ET-1. ADP-induced aggregation was not significantly altered. The specificity of the inhibition of adrenaline induced secondary aggregation, is consistent with the findings presented in chapter 3 and indicates an attenuation of a mechanism of secondary aggregation response not present in ADP induced aggregation.

Pre-treatment with aspirin significantly potentiated the vasoconstriction elicited by ET-1, suggesting a role for vascular generation of vasodilator prostanoids in the modulation of ET-1 induced vasoconstriction in the human forearm resistance vessels. The inability of aspirin to influence basal resistance (Linder *et al.*, 1990) would indicate that prostanoids are not produced basally, in contrast to NO (Vallance, 1989). Consequently, one can conclude that ET-1 stimulates prostanoid generation. This is consistent with *in vitro* evidence that ET-1 stimulates prostacyclin generation from cultured endothelial cells (Filep *et al.*, 1991). ET-1 also stimulates the generation of prostaglandin E<sub>2</sub> (Miura *et al.*, 1991) and thromboxane A<sub>2</sub> (De Nucci *et al.*, 1988). However, the fact that aspirin caused increased constriction to ET-1 would indicate that any generation of thromboxane is less important than prostanoid induced dilation in the presence of a healthy endothelium. These findings are consistent with the findings of Haynes (Haynes & Webb, 1993b) that venoconstriction to ET-1 is modulated by prostanoids in the human hand vein.

The ET-1 mediated inhibition of adrenaline-induced aggregation is consistent with the results presented in chapter 3. However, in the *ex vivo* studies presented in this chapter the platelets were exposed to ET-1 an hour before being challenged with adrenaline. This would imply that the effect of ET-1 on platelets is sustained for a prolonged period after the initial exposure. This is analogous to the prolonged vasoconstriction observed in the forearm, discussed above. It is an interesting part of the profile of the actions of ET-1 that it induces such a sustained effect.

Hermán (Hermán, *et al.*, 1989) reported that ET-1 mediated inhibition of *ex vivo* platelet aggregation in dogs was accompanied by an increase in 6-keto PGF<sub>1α</sub>, the hydrolysis product of prostacyclin. In Hermán's study inhibition of aggregation was inhibited by aspirin. From this he concluded that prostacyclin was involved in ET mediated inhibition of platelet aggregation. To investigate the role of prostacyclin in the actions of ET-1 in man, in one phase of the study presented in this chapter, subjects were given aspirin. However, in addition to blocking endothelial prostanoid production, aspirin also inhibits platelet thromboxane A<sub>2</sub> production. Thromboxane A<sub>2</sub> production is one of the mechanisms that promotes secondary aggregation to adrenaline, and after receiving aspirin, secondary aggregation to adrenaline was only observed in plasma from 3 of the 12 subjects. Therefore, no statistical analysis could be performed. Secondary aggregation to ADP in this study, was both aspirin insensitive and ET-1 insensitive. This might suggest that ET-1, like aspirin, alters platelet thromboxane production. However, this would be better investigated by measuring thromboxane B<sub>2</sub>, a stable metabolite of thromboxane A<sub>2</sub>, levels after *in vitro* exposure of platelets to ET-1. Recent work has provided evidence that rather than altering thromboxane A<sub>2</sub> production, ET-3 attenuates Ca<sup>2+</sup> mobilisation from internal stores linked to thromboxane receptor stimulation (Astarie-Dequeker *et al.*, 1995). Significantly, with a regards to the lack of effect of ET-1 on ADP-induced



secondary aggregation, the same publication reported that ET-3 did not affect  $\text{Ca}^{2+}$  changes induced by ADP.

The fact that prostacyclin is not responsible for ET-1 inhibition of platelet aggregation is better demonstrated in the *in vitro* phase of the study. Platelets do not produce prostacyclin and it has been reported that the anti-aggregatory activity of prostacyclin is 'completely diminished' after 20 min (Moncada, *et al.*, 1976). As the studies in this chapter were performed at least 60 min after venesection, it is unlikely that prostacyclin was involved in the inhibition of aggregation observed after the exposure to ET-1.

No significant potentiation of aggregation was observed in any of the phases of the study presented in this chapter, although there was a non-significant trend for ET-1 to potentiate primary aggregation in the *in vitro* phase. It is possible that the concentration of ET-1 used was not sufficient. However, in chapter 4, a slight but significant potentiation was observed at the same concentration of ET-1 (100 pM). The *in vitro* concentration of 100 pM was used because this was estimated to be approximately the same concentration as would be achieved *in vivo* during the *ex vivo* phases of the study. It might have been useful to have also used a higher concentration *in vitro* to confirm that the subjects studied did, indeed, display a potentiating effect, in light of the suggestions in earlier chapters that this effect might not be seen in all individuals. In this particular study it would not have been practicable to have done this as it would not only have required more blood but would also have extended the duration of the study beyond the time established in chapter 2 for achieving reproducible results for adrenaline-induced aggregation.

As mentioned in the preceding paragraph, the concentration of ET-1 achieved *in vivo* after infusion was approximately 100 pM. This was estimated on the basis that

resting FBF is approximately  $50 \text{ ml min}^{-1}$ , and thus a dose of  $5 \text{ pmol min}^{-1}$  would achieve local arterial concentrations of 100 pM. However, venous plasma ET concentrations, as determined by radioimmunoassay, were only raised to 15 pM. In addition to neutral endopeptidase breakdown Plumpton suggested that other mechanisms may be involved in the control of circulating concentrations of ET-1 (Plumpton *et al.*, 1995). Rapid internalisation of receptor bound ET-1 by arterial and nutritive vessels (Anggard *et al.*, 1989) may be one possible mechanism. The combination of ET-1 metabolism and internalisation of receptor bound ET-1 might account for the discrepancy between this result and the expected value.

The discrepancies between the results presented in this chapter and other studies on the role of prostacyclin in modulating the effect of ET-1 on *ex vivo* platelet aggregation may be due to a number of factors. In addition to possible interspecies variation, the most significant methodological differences are the fact that other workers used systemic concentrations of ET-1 and studied different aggregating agents. Many of the reports, including those by Lidbury and Thiemermann (Lidbury, *et al.*, 1989; Thiemermann, *et al.*, 1989), only described the use of ADP as the platelet aggregating agent. As discussed above, ADP-induced aggregation appears to be ET insensitive.

In summary, endogenous endothelium derived dilator prostanoid production limits the vasoconstriction observed in response to exogenous ET-1 in the human forearm. ET-1 inhibits *ex vivo* and *in vitro* platelet aggregation to adrenaline but not to ADP. The *ex vivo* inhibitory effect was long lasting, still present two hours after the infusion of ET-1 was complete.

	Forearm Blood Flow		Platelet Aggregation		MAP	HR
	Control	Infused	Control	Infused		
	Arm	Arm	Arm	Arm		
- Asp.	3.1 ± 0.7	3.0 ± 0.6	2.3 ± 0.3	2.4 ± 0.3	80.4 ± 3.0	62.6 ± 2.4
+ Asp	2.4 ± 0.2	2.9 ± 0.2	2.1 ± 0.3	2.0 ± 0.3	84.7 ± 2.6	59.1 ± 2.2

**Table 7.1.** This table provides a comparison of the mean basal data for forearm blood flow, platelet aggregation, mean arterial pressure (MAP) and heart rate (HR) on the days without (- Asp) and with (+ Asp) aspirin administration. Mean forearm blood flow is given for pre-ET-1 infusion and mean platelet aggregation with the *in vitro* addition of vehicle only.

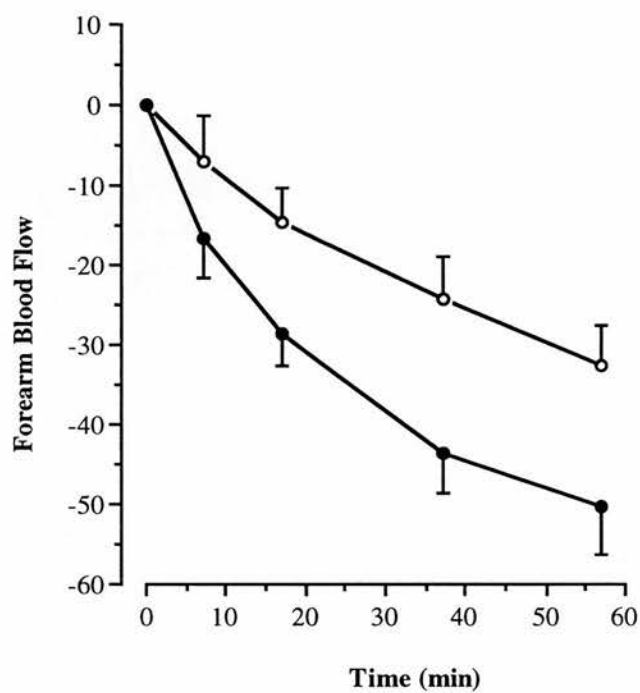


Figure 7.1. Forearm blood flow responses to ET-1, 5 nM infused at a rate of 1 ml min<sup>-1</sup> for 60 min, n=12, in the absence (○) and presence (●) of intravenous aspirin infusion. ET-1 reduced forearm blood flow compared to baseline  $P < 0.0001$  for the ET-1 response, Aspirin significantly potentiated ET-1 induced vasoconstriction  $p < 0.05$

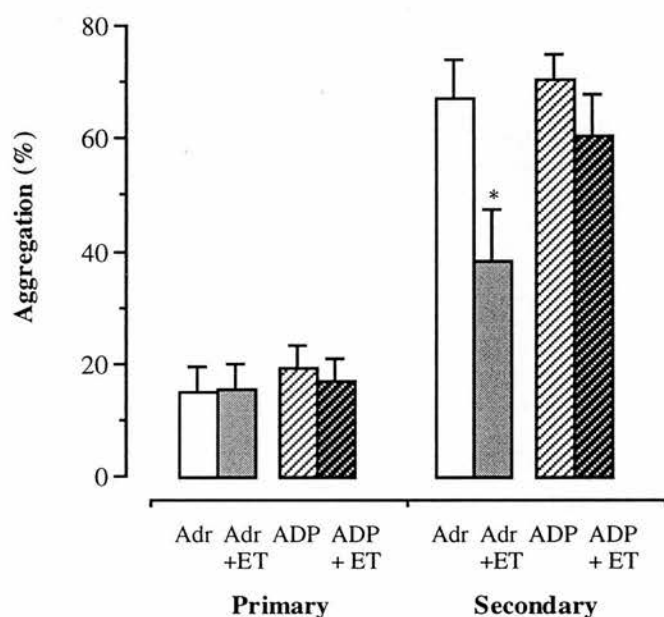


Figure 7.2. Primary and secondary aggregatory responses to adrenaline (adr) ■ and ADP ■ of platelets from either the control arm or the infused arm, ET-1, adr from infused arm ▨, ADP from infused arm ▩ n=12. ET-1 significantly inhibited adrenaline induced secondary aggregation  $p < 0.05$ .

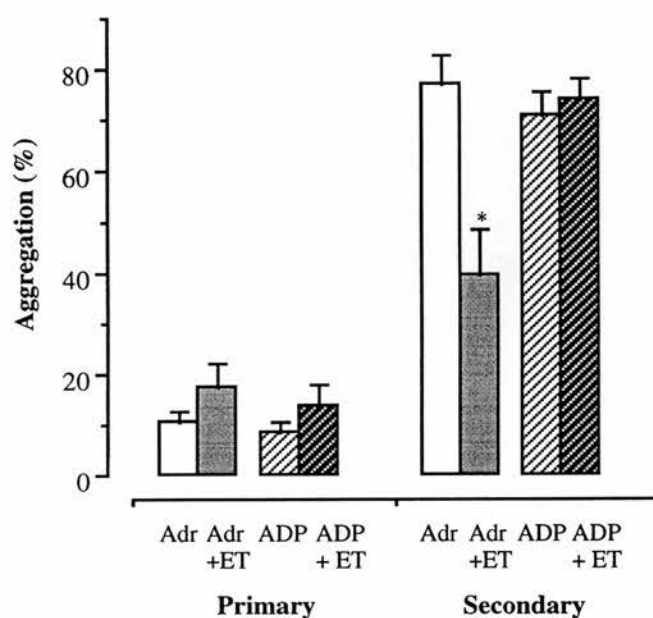


Figure 7.3 Primary and secondary aggregatory responses to adrenaline (adr) ■ and ADP ■ pre-incubated *in vitro* with either vehicle or ET-1 (0.1 nM) adr + ET-1 ■, ADP + ET-1 ■ n=12. ET-1 significantly inhibited adrenaline induced secondary aggregation  $p<0.05$ .

## **CHAPTER 8**

### **ABNORMAL ENDOTHELIN-1 MEDIATED RESPONSES IN SUBJECTS WITH HIGH BLOOD PRESSURE**



## INTRODUCTION

In the original identification of ET Yanagisawa and colleagues suggested that 'disturbances in the control of ET production might contribute to the pathogenesis of hypertension' (Yanagisawa, *et al.*, 1988). Indeed, the potent vasoconstrictor properties of ET-1 and its potential for increasing peripheral vascular resistance, suggest that it might have a role in the maintenance of blood pressure. However, plasma concentrations are not raised in patients with essential hypertension compared to age matched controls (Davenport, *et al.*, 1990). This would not necessarily rule out a role for ET-1 in hypertension for at least three reasons. (1) circulating plasma levels of ir-ET may not represent local production of ET, (2) increased sensitivity to ET could occur by increased receptor number or increased post receptor activity, and (3) a decrease in the ET mediated counter-balancing dilator activity of agents such as NO. All of these, either separately or in combination, might result in increased blood pressure. Although studies which have examined increased sensitivity to ET-1 in animal models of hypertension are inconclusive (Rubanyi & Polokoff, 1994), an increased sensitivity to exogenous ET-1 has been demonstrated in dorsal hand veins of untreated hypertensive patients compared to normotensive subjects (Haynes *et al.*, 1994).

The work reported in earlier chapters of this thesis suggests that ET-1 sensitivity could also be examined in the platelets of patients with hypertension. Platelets may play a role in the pathophysiology of hypertension by the release of several vasoactive agents, including thromboxane A<sub>2</sub>, serotonin, ADP and platelet derived growth factor from activated platelets. A number of functional abnormalities have been described in the platelets of patients suffering from essential hypertension. These include increased sensitivity to several platelet activating agents (De Clerck, 1986; Vlachakis & Aledort, 1980), increased platelet [Ca<sup>2+</sup>]<sub>i</sub> (Le Quan Sang &

Devynck, 1986) and raised intracellular pH (Astarie *et al.*, 1992). In chapter 3 and 4 of this thesis evidence was presented for a potentiation of adrenaline-induced aggregation by ET-1 and an increased sensitivity to ET-1 in hypertensive subjects could result in increased platelet aggregation. Alternatively, an ET mediated inhibition of platelet aggregation has also been described both *ex vivo* and *in vitro* in chapter 7. Although the precise mechanism of this inhibitory action is not known, a reduction in this inhibition may also result in increased platelet aggregation. The aim of the work presented in this chapter was to examine whether platelet sensitivity to ET receptor agonists is altered in subjects with high blood pressure. In order to investigate this two separate studies were carried out.

Platelet responses were studied in patients with untreated hypertension and age and sex matched controls to examine whether platelet responses to ET-1 were altered in subjects with established hypertension. Platelet aggregation was studied in response to adrenaline in the presence and absence of ET-1. Platelet cyclic nucleotides were also measured in response to adrenaline, ET-1 and sarafotoxin S6c.

Studies of platelet responses in patients with hypertension may provide evidence of an association of hypertension and aberrant platelet activity, and may provide information on a potential role of platelets in the consequences of this condition. However, they do not necessarily establish whether altered platelet activity is a cause or a consequence of the hypertension. In an attempt to distinguish between the two a separate study was carried out using subjects from a 'four corners' epidemiological study. In this 'four corners' study young adult subjects were identified as having either high or low blood pressure, in addition they were also classified according to parental blood pressure. Hence, subjects with a predisposition to hypertension, on the basis of having raised blood pressure, can be identified and distinguished from subjects with a familial predisposition to hypertension, that is, subjects with raised

blood pressure who also have parents with raised blood pressure. Consequently, it should be possible to identify effects which may be involved in the development of hypertension. In this study, adrenaline-induced platelet aggregation and the effect of ET-1 on adrenaline-induced primary and secondary aggregation were studied.

Adrenaline has been chosen as the platelet aggregating agent for both of the studies presented in this chapter. In previous chapters ET-1 mediated modulation of platelet aggregation has been described to adrenaline but not to ADP. In addition adrenaline may be a particularly appropriate aggregating agent to study as there is considerable evidence for altered peripheral adrenergic receptor activity in hypertension (Michel *et al.*, 1990).

## METHODS

### Study 1.

Ten untreated patients with essential hypertension and 10 age and sex matched normotensive control subjects were recruited. Age and blood pressure data is given in table 8.1. Venous blood samples were collected and plasma was prepared as described in chapter 2. A preliminary dose response to adrenaline was performed for each subject and subsequently the effect of pre-incubation with vehicle or ET-1 ( $10^{-11}$ - $10^{-8}$  M) on adrenaline-induced primary and secondary aggregation was examined.

Cyclic nucleotide measurements were carried out as described in chapter 2. Briefly, PRP was pre-incubated for 3 min at 37°C before the addition of adrenaline (0.3-0.03  $\mu\text{g ml}^{-1}$ ), ET-1 ( $10^{-11}$ - $10^{-7}$  M), SRTX S6c ( $10^{-11}$ - $10^{-7}$  M) or vehicle (0.9% saline). The reaction was stopped after a further 5 min by centrifugation at 5°C. The platelet pellet was isolated and cyclic nucleotides were extracted using ethanol. Samples were dried down at 55°C and the residue resuspended in assay buffer and frozen until required for assay. Acetylated samples were assayed for cyclic AMP and cyclic GMP.

### Study 2

In 1977, in the screening for the MRC Mild Hypertension Trial blood pressure was measured in 603 couples at the Ladywell Medical Centre. In 1985 blood pressure was measured in 864 of their offspring, aged 16-24 years. Offspring blood pressure was plotted against mean parental blood pressure using age adjusted Z-scores. These were divided into tertiles, thus defining members of 4 extreme corners: A low parental/high offspring; B high parental/high offspring; C low parental/low

offspring; and D high parental/low offspring(Watt *et al.*, 1992). Eleven male subjects drawn at random from each corner were studied.

Blood was collected in sodium citrate to give a final concentration of 3.5% and centrifuged at 800 *g* for 5 min to produce platelet rich plasma (PRP). A 2 ml aliquot of PRP was centrifuged at 3000 *g* to give platelet poor plasma. The remaining PRP was divided into 12 aliquots of 890  $\mu$ l and stored under 95% O<sub>2</sub>:5% CO<sub>2</sub> at room temperature until required, but for no longer than 120 min. For the aggregation studies, PRP was incubated at 37°C for 3 min before the addition of either vehicle or ET-1, (1 nM). After 1 min a second addition of vehicle or adrenaline, (0.5 to 5.0  $\mu$ M) was made.

Comparisons for dose responses to adrenaline alone between the 4 corners was by repeated measures ANOVA. The effect of ET-1 on adrenaline-induced primary and secondary aggregation was calculated by paired Student's *t* test.

## RESULTS

### Study 1

#### *Platelet Aggregation*

Adrenaline induced dose dependent platelet aggregation in plasma from both the hypertensive group and the age/sex matched controls ( $p=0.0001$  for both), with no difference between the groups (Fig. 9.2).

ET-1 potentiated primary aggregation in a dose dependent manner in both groups ( $p= 0.002$  for the hypertensive group and  $p= 0.04$  for the control group) with no difference between groups (Fig. 9.3). Adrenaline-induced secondary aggregation was

significantly inhibited by ET-1 in the control group ( $p=0.001$ ) but not in the hypertensives ( $p=0.41$ ) (Fig. 9.4).

#### *Platelet Cyclic Nucleotide Measurement.*

Basal platelet cyclic AMP levels were not significantly different between the groups. Treatment with adrenaline, ET-1 and SRTX S6 did not significantly affect platelet cyclic AMP levels in either group (data not shown), this is consistent with the findings reported in chapter 5.(see figure 5.2).

Basal cyclic GMP levels were not significantly different between the groups. Adrenaline produced a change in platelet cyclic GMP in both groups of borderline statistical significance ( $p=0.05$ , for both the hypertensive group and the controls) (Fig. 9.5). However the apparent difference between the groups was not attributable to the increasing concentration of adrenaline, as determined by repeated measure ANOVA of the difference between the groups ( $p= 0.23$ ).

ET-1 did not induce a statistically significant change in cyclic GMP in either group (Fig 9.6). However, SRTX S6c did produce a dose-dependent accumulation of cyclic GMP in platelets from the control group ( $p= 0.04$ ), but a non-significant decrease in cyclic GMP in platelets from the hypertensive group ( $p= 0.07$ ) (Fig 9.7). The platelet cyclic GMP response to SRTX S6c was significantly greater in the normotensive group than in the hypertensives ( $p=0.009$ ).

#### **Study 2.**

Adrenaline elicited dose dependent platelet aggregation in all 4 groups (Fig 9.8,  $p=0.001$  for all groups). Analysis by 2 factor repeated measure ANOVA indicated a difference in the response to adrenaline between groups, ( $p=0.03$ ). This difference



appeared to be due to the difference in response to adrenaline between corners A and C, ( $p=0.003$ ). The mean response also tended to be greater in corner C than in B and D but this did not reach statistical significance, ( $p=0.07$  and  $0.18$  respectively).

Pre-incubation with ET-1 tended to increase adrenaline-induced primary aggregation in all groups (Fig 9.9). However, this only reached statistical significance in group B ( $p=0.04$ ). Inhibition of adrenaline-induced secondary aggregation by ET-1 did not achieve statistical significance in any of the corners, although a consistent tendency was observed in all 4 corners. When the results from all 4 corners were taken together ET-1 significantly potentiated primary aggregation ( $p=0.02$ ), but inhibition of secondary aggregation was not significant ( $p=0.10$ ).

## DISCUSSION

In study 1, no difference was observed between the hypertensive subjects and the normotensive controls in the aggregatory response to increasing concentrations of adrenaline, or the potentiation of adrenaline-induced primary aggregation by ET-1. However, platelets from the hypertensive subjects failed to exhibit a significant ET mediated inhibition of secondary aggregation observed in platelets from the normotensive subjects. This is consistent with the findings of Touyz and Schiffrin, who reported an ET-1 mediated inhibition of thrombin induced platelet aggregation in normotensive but not hypertensive subjects (Touyz & Schiffrin, 1993b).

The study of platelet cyclic GMP produced two interesting differences between the hypertensive and normotensive subjects. Adrenaline stimulated an increase in platelet cyclic GMP. As can be seen in figure 8.5 the levels in the normotensive subjects appear to be almost double that in the hypertensives. However, by analysing differences between the groups it can be seen that the difference does not increase

with increasing concentrations of adrenaline and, therefore, one can conclude that the difference between the groups is not related to the increasing concentration of agonist, but is probably related to a generalised impairment of cyclic GMP production in the hypertensive subjects. The fact that basal cyclic nucleotide levels were not different suggests that there is an impairment of stimulated cyclic GMP production or an increase in phosphodiesterase activity leading to less cyclic GMP remaining in the platelet. Further cyclic nucleotide studies, in the presence of phosphodiesterase inhibitors, would have made it possible to distinguish between increased cyclic nucleotide production and altered cyclic nucleotide metabolism. Cyclic GMP is associated with both vasodilatation and inhibition of platelet activity, and reduced cyclic GMP levels may be a significant factor in hypertension.

ET-1 appeared to raise platelet cyclic GMP in the normotensive subjects as can be seen in figure 8.8. However, this was not statistically significant. SRTX S6c did produce a significant rise in cyclic GMP levels in the normotensive but not the hypertensive subjects. Unlike the effect of adrenaline, the difference observed between the two groups of subjects with SRTX S6c is related to increasing agonist concentration. Hypertensive patients did not show an ET receptor mediated inhibition of aggregation, nor did they show SRTX S6c stimulated accumulation of cyclic GMP. However, the inhibition of platelet aggregation mediated by ET receptors may not be solely mediated by an increase in cyclic GMP because ET-1 inhibited platelet aggregation at  $10^{-8}$  M without significantly affecting cyclic GMP levels. A further experiment which may shed light on the role of cyclic GMP in ET receptor mediated inhibition of platelet aggregation would be to perform aggregation studies with ET-1 and SRTX S6c in the presence and absence of either a guanylate cyclase inhibitor or an inhibitor of cyclic GMP dependent protein kinase. The fact that SRTX S6c appeared to have a greater effect than ET-1 may be an important observation. It is consistent with the trends reported in earlier chapters, where the



effect of the ET<sub>B</sub> receptor agonist appeared to be greater than ET<sub>A/B</sub> agonist on platelet aggregation and cyclic GMP accumulation. This possibly reflects opposing effects mediated by different platelet ET receptors, and will be discussed in the following chapter.

Study 2 identified an altered platelet response to adrenaline between the four corners of the Ladywell study. Interestingly, the corner that displayed the greatest aggregatory response was corner C, the group representing subjects with low blood pressure whose parents have low blood pressure. The least aggregatory response was seen in the subjects from corner A, the group representing subjects with high blood pressure from parents with low blood pressure. This would suggest that *in vitro* platelet responsiveness to adrenaline can be determined by the subject's blood pressure and is not dependent on parental blood pressure. Study 2 also demonstrated a difference between the four corners on the potentiation of primary aggregation by ET-1. Corner B was the only group that displayed a significant potentiation of adrenaline induced aggregation. The fact that corner A did not show a significant potentiation suggests that this effect is not related to high blood pressure *per se* but possibly to a genetic element of raised blood pressure. In addition, the dose response to adrenaline was similar for these two corners reinforcing the proposition that the effect is ET-1 dependent.

The fact that subjects from corner C displayed the greatest aggregatory response to adrenaline was an unexpected finding. Some studies which have examined the relationship between platelet aggregation and blood pressure would suggest that increased blood pressure would be associated with increased platelet activation. A study which examined the relationship between platelet free calcium and blood pressure found that the two factors were positively correlated in both hypertensive and normotensive subjects (Erne *et al.*, 1984). In addition, Brodie reported a positive

correlation between mean arterial pressure and adrenaline-induced platelet aggregation (Brodde *et al.*, 1985). However, in this latter study they found that some hypertensive patients had normal responses while others had increased responses, and they suggested that the degree of hypertension was an important factor. Many factors that are associated with increased vascular resistance are also associated with increased platelet activation, as discussed in chapter 1. However, the exception to this rule may be cyclooxygenase activity.

In the vasculature, the predominant result of cyclooxygenase activity is the production of the vasodilator prostacyclin. Conversely, in the platelets the main active products, such as thromboxane A<sub>2</sub>, are pro-aggregatory. Although this is speculation, it may have been interesting to examine cyclooxygenase activity in these subjects. From this work it is not possible to know whether the effects observed are due to differences specifically related to adrenaline, such as  $\alpha_2$  receptor density, or platelet responsiveness in general. It would, therefore have been useful to have employed more than one platelet aggregating agent. Additionally, measurement of *in vivo* markers of platelet activation such as thromboxane B<sub>2</sub>, the stable metabolite of thromboxane A<sub>2</sub> and PGF<sub>2</sub> $\alpha$ , the metabolite of prostacyclin, would have helped identify both the *in vivo* activity of platelets and endothelial cyclooxygenase activity.

The two studies presented in this chapter were designed to examine the effect of blood pressure and essential hypertension on endothelin modulation of platelet aggregation and the possible role of platelet aggregation on the development of high blood pressure. There is however, an apparent inconsistency in the results obtained. In study 1 both groups showed potentiation of adrenaline-induced primary aggregation by endothelin-1, whereas in study 2 only 1 of the 4 corners demonstrated potentiation. An important element here is that the subjects in study 2

do not have hypertension and the data may reflect variability in the effect of endothelin-1 observed with healthy control subjects in previous chapters. Although study 2 was designed to study differences between the 4 corners, it is worth noting that when the effect of ET-1 on adrenaline-induced primary aggregation is calculated for all four corners together as a single population a significant potentiation is seen.

In addition to this the subjects studied and the precise method employed in the preparation of the plasma were different. Study 1 used subjects of both sexes with ages as high as 63 years whereas in study 2 the subjects were young males. In study 1 PRP was prepared by centrifugation at 120 g for 10 min, in keeping with the methods used in studies reported in previous chapters, whereas in study 2 PRP was prepared by centrifugation at 800 g for 5 min. This difference was due to the fact that in study 2 the plasma was prepared for use in a number of different studies. The result is that denser, and potentially more active platelets may not have been collected. Consequently, although comparisons within the studies remain valid, comparisons between the two studies may be inappropriate. It would have been useful if platelet size profiles had been carried out in plasma prepared by centrifugation at 800 g for 5 min and compared with those obtained by centrifugation at 120 g for 10 min.

Where study 1 was designed to examine and compare platelet responses between subjects with established hypertension with age matched controls, study 2 was designed to look at subjects who possibly had a familial predisposition to hypertension, corner B, compared to those who may be predisposed to hypertension by simply having raised blood pressure, corner A. Consequently, the hypertensive subjects involved in study 1 are probably a heterogeneous population involving subjects with a clear familial predisposition and those without (that is a combination

of individuals who would fall into corner A and B). It is also possible that sustained raised blood pressure may itself cause altered platelet activity.

From these studies it is possible to draw three conclusions. First, in essential hypertension, a significant ET mediated inhibition of secondary platelet aggregation is not observed. Secondly, platelets from subjects with essential hypertension have a reduced response to SRTX S6c mediated increase in platelet cyclic GMP. Thirdly, potentiation of adrenaline-induced primary aggregation occurs in subjects who are predisposed to hypertension by virtue of raised blood pressure and familial factors but not in those with raised blood pressure alone, suggesting a genetic component to increased sensitivity to ET.

	Normotensive Subjects	Hypertensive Subjects
Age (years)	48 ± 3	48 ± 3
Sex (M:F)	7:3	7:3
Blood Pressure (mm Hg)		
Systolic	119 ± 3	172 ± 5
Diastolic	74 + 3	105 ± 1

Table 8.1a Data for subjects in study 1

Corners	A	B	C	D
Age (years)	28 ± 1	29 ± 1	30 ± 1	28 ± 1
Sex (M:F)	11:0	11:0	11:0	11:0
Blood Pressure (mm HG)				
Systolic	117 ± 2	118 ± 3	112 ± 2	108 ± 1
Diastolic	76 ± 1	80 ± 2	72 ± 2	72 ± 1

Table 8.1b Data for subjects in study 2

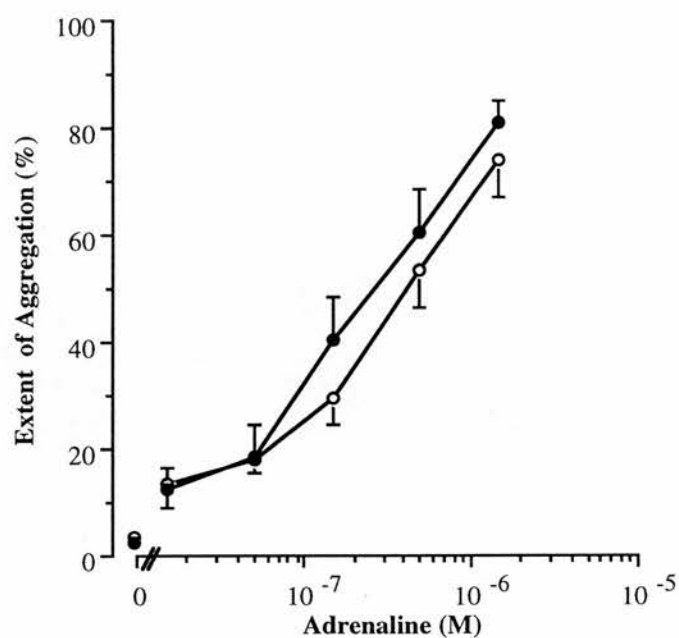


Figure 8.2 Aggregatory response to adrenaline for normotensive subjects (o) and hypertensive subjects (●) .

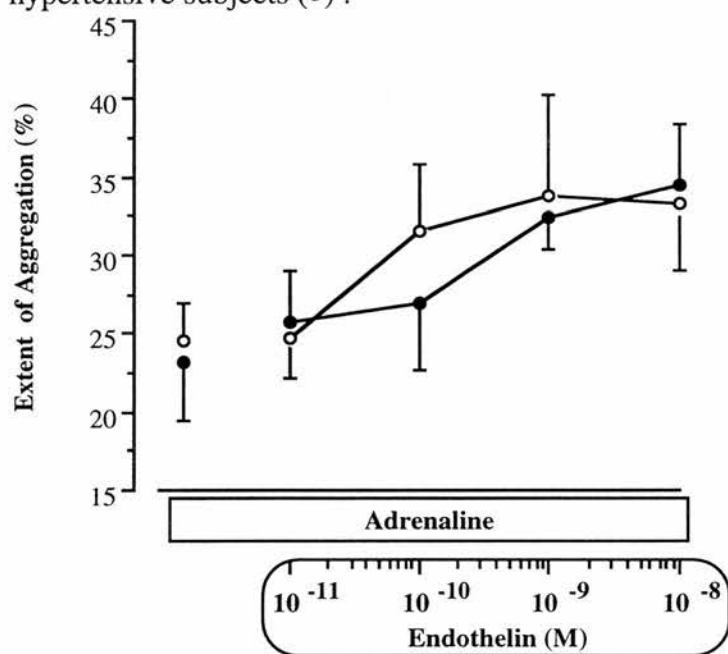


Figure 8.3 The effect of ET-1 on adrenaline-induced primary aggregation in normotensive subjects (o) and hypertensive subjects (●) .

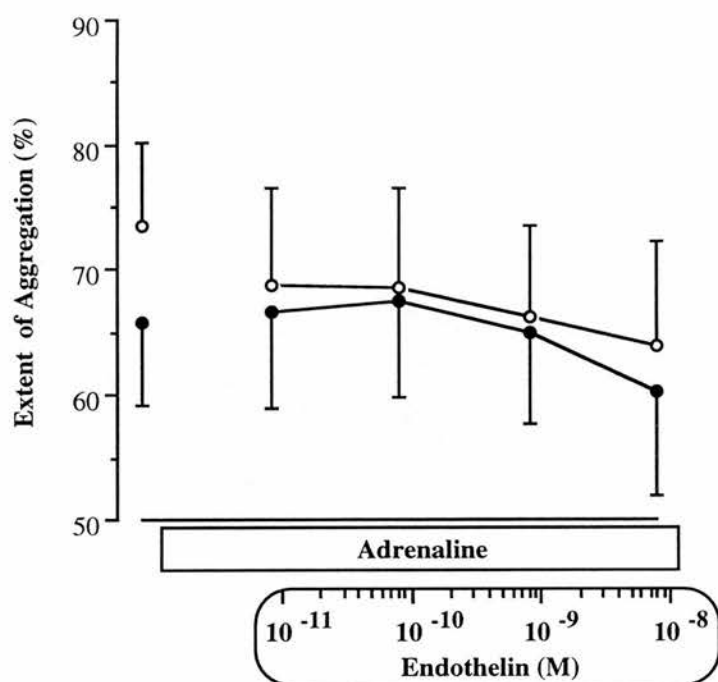


Figure 8.4 The effect of ET-1 on adrenaline-induced secondary aggregation in normotensive subjects (°) and hypertensive subjects (•).

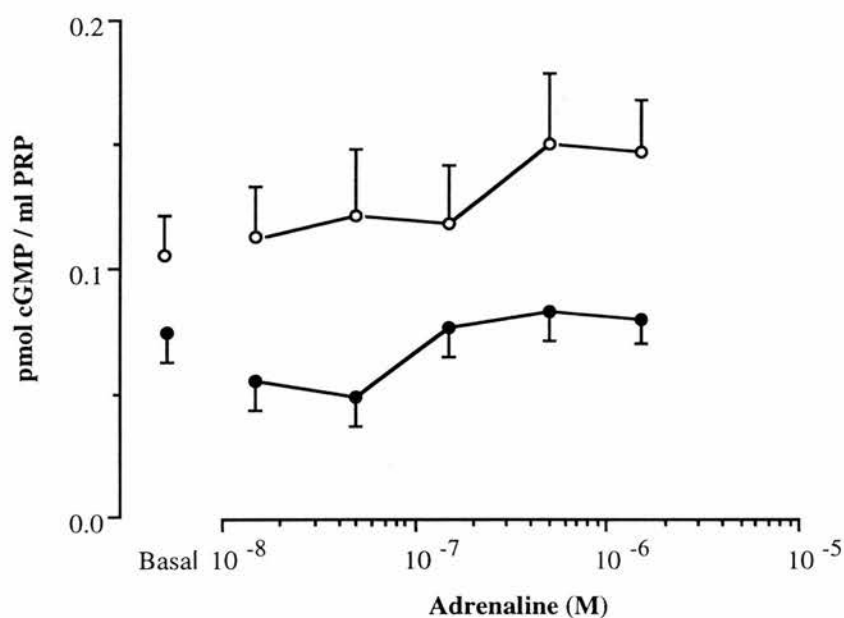


Figure 8.5 The effect of adrenaline on platelet cyclic GMP concentrations in normotensive subjects (°) and hypertensive subjects (•).



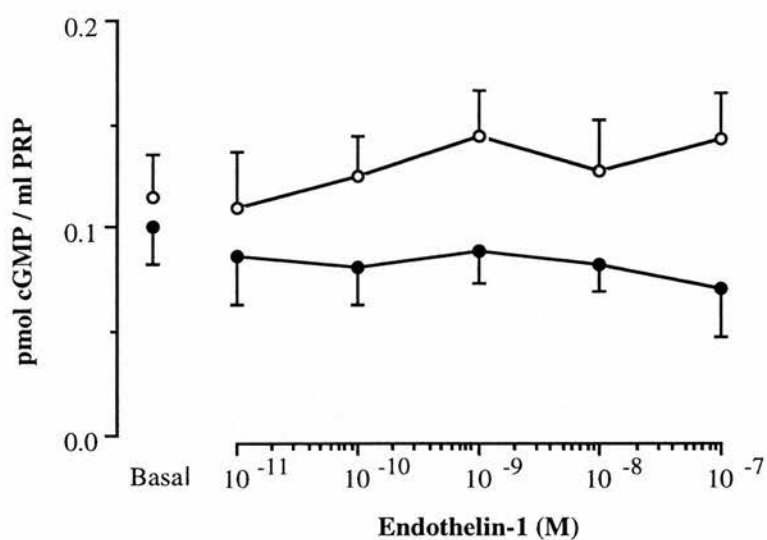


Figure 8.6 The effect of ET-1 on platelet cyclic GMP concentrations in normotensive subjects (○) and hypertensive subjects (●) .

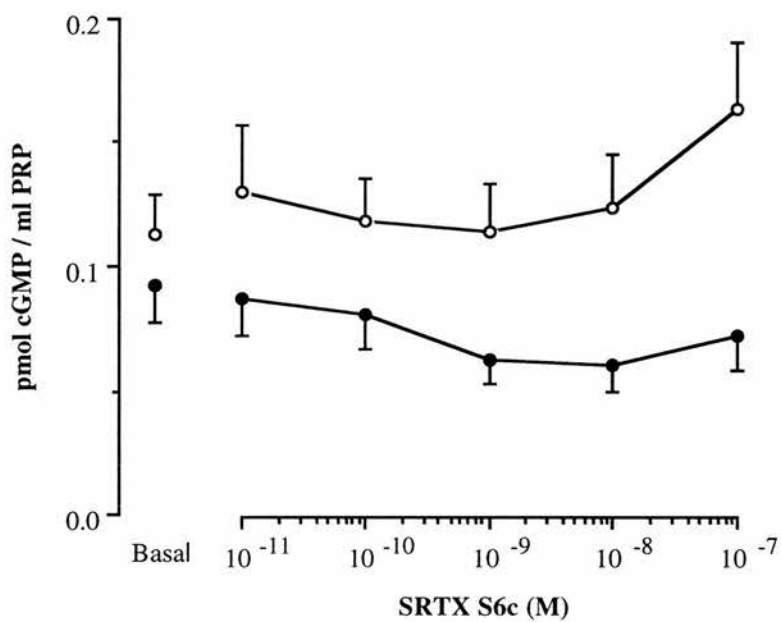


Figure 8.7 The effect of SRTX S6c on platelet cyclic GMP concentrations in normotensive subjects (○) and hypertensive subjects (●) .

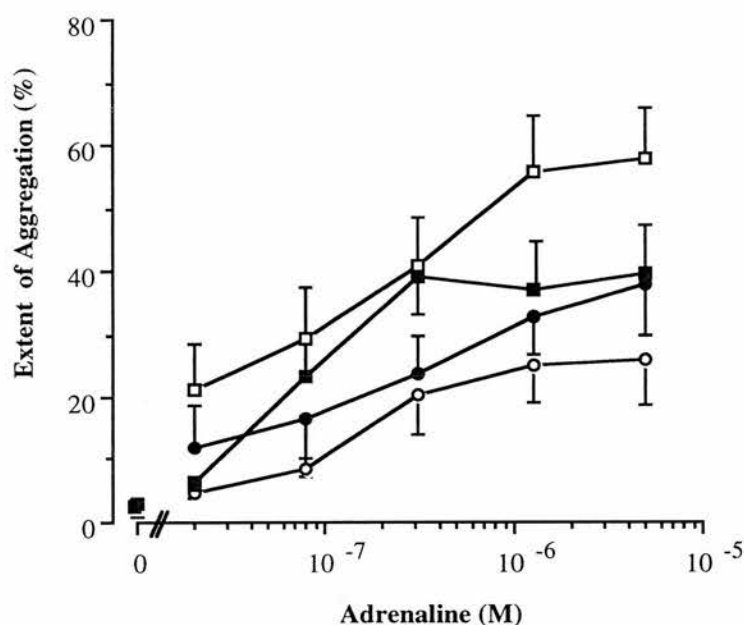


Figure 8.8 Aggregatory response to adrenaline in samples from the 4 corners of study 2 corner A (○), corner B (●), corner C (□) and corner D (■).

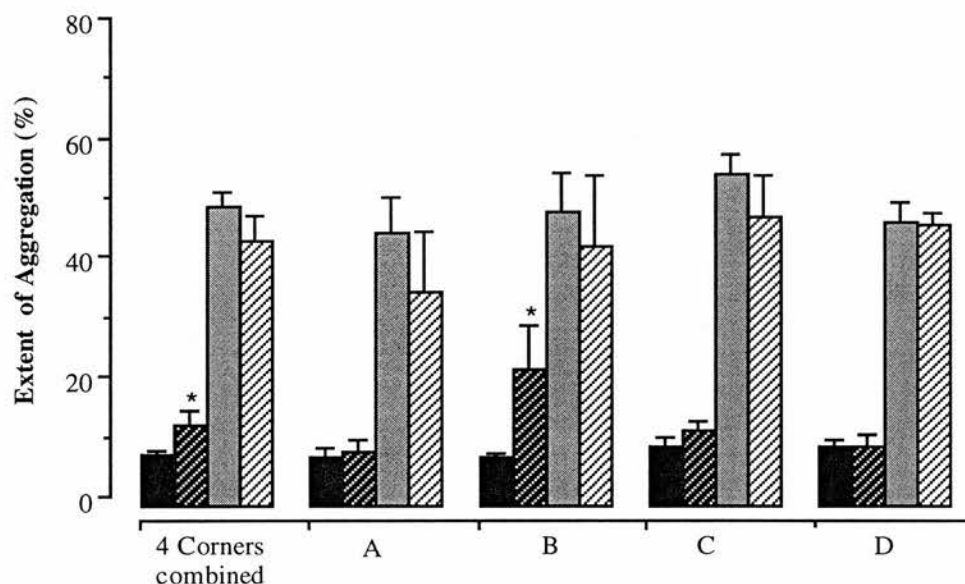


Figure 8.9 The effect of ET-1 (1 nM) on adrenaline-induced primary and secondary aggregation in the 4 corners of study 2. Adrenaline-induced primary aggregation (■), the same concentration of adrenaline in the presence of ET-1 (▨) Adrenaline-induced secondary aggregation (■), the same concentration of adrenaline in the presence of ET-1 (▨). \* indicates  $p < 0.05$  compared to adrenaline alone.

## **CHAPTER 9**

### **DISCUSSION AND CONCLUSION**

In the introduction to this Thesis three main aims were set out: to establish whether ET-1 acts directly on human platelets, to characterise the receptors involved, and to investigate the actions of ET-1 on platelets in diseases where ET-1 is thought to have a pathophysiological role. These aims were largely fulfilled in the experiments described in chapters 3 to 8.

In chapter 3, investigative studies were described and a number of conclusions were reached. ET-1 alone caused a slight but statistically significant aggregation of platelets, which in isolation is unlikely to be of any substantial biological significance. Perhaps of more physiological importance, ET-1 modulated the actions of adrenaline but not ADP, potentiating primary aggregation and inhibiting secondary aggregation. The ET-1-mediated potentiation of adrenaline-induced primary aggregation was consistently significant in all three studies described in this chapter. However, the inhibition of aggregation only achieved statistical significance in one of the two studies in which it was examined. From this it appeared that the pro-aggregatory effect of ET-1 was specific to the aggregating agent used. However, other 'weak' agonists that are reported to produce a quantifiable primary phase of aggregation were not studied and it would be inappropriate on the evidence to conclude that the potentiating effects of ET-1 are specific to adrenaline. Other 'weak' physiologically important agonists that could have been studied include angiotensin II and serotonin. However, in the absence of another aggregating agent angiotensin II does not appear to cause platelet aggregation and the extent of serotonin-induced aggregation was so low that it was, at this time, not considered a useful agent to use in these preliminary studies. Further work might consider the interactions of one or more of these modulators of platelet aggregation. As described in chapter 1 secondary aggregation to agonists such as adrenaline and ADP is dependent on a positive feedback mechanism involving amplification of the original signal and the

release of platelet granules. Possible differences between ADP and adrenaline induced aggregation are discussed below.

The receptor mediating the pro-aggregatory effect of ET-1 was characterised in the studies described in chapter 4. Adrenaline-induced primary aggregation was significantly potentiated by the non-selective receptor agonist, ET-1 and the ET<sub>B</sub> selective agonist SRTX S6c. The response to both ET receptor agonists appeared to vary within the subject group and, although not statistically different, SRTX S6c appeared to have a greater effect than ET-1. Both of these trends were also observed in studies described in other chapters. The pro-aggregatory effect of ET-1 was inhibited by the ET<sub>B</sub> antagonist, BQ-788, but not the ET<sub>A</sub> antagonist BQ-123. From these results it was concluded that, the pro-aggregatory effect of ET-1 on human platelets was primarily mediated by the ET<sub>B</sub> receptor.

In the antagonist studies described in chapter 4 only samples where a potentiation of aggregation by ET-1 was observed were used. Although the number of subjects studied was not large enough to establish a bimodal distribution of response, where potentiation was observed it was seen to be reproducible. It was thought that including all subjects, where the mean potentiation to ET-1 though significant was relatively small (see figure 4.1b), might have made it difficult to discern significant inhibition by the antagonist, and therefore draw a clear conclusion.

In the studies reported in chapter 5, the effects of ET-1 and SRTX S6c on platelet cyclic AMP and cyclic GMP were examined. Platelet cyclic AMP levels were not significantly affected by either agonist. However, both agonists caused a dose dependent accumulation of platelet cyclic GMP. As for studies described in chapter 4, the effect of both agonists was variable and SRTX S6c again appeared to have a greater effect. However, as in chapter 4, there was no statistical difference between

the effects of the two agonists. These results suggest that an ET receptor is associated with the accumulation of platelet cyclic GMP and that this receptor is probably of the ET<sub>B</sub> type. As these experiments were carried out in the absence of phosphodiesterase inhibitors it is not possible to conclude whether the accumulation of cyclic GMP is due to increased guanylate cyclase activity or decreased phosphodiesterase activity. Nor is it possible, on the basis of the studies presented in this chapter, to associate the change in cyclic GMP levels with a particular directional modulation of aggregation. An increase in cyclic GMP is normally associated with an inhibition of platelet aggregation and that might suggest that the inhibitory actions of ET are also mediated by the ET<sub>B</sub> receptor. However, the study reported in chapter 8 is better designed to interpret this relationship as the modulation of platelet aggregation and the effect on platelet cyclic nucleotides were examined together.

Platelets contain a number of intracellular second messengers, including inositol trisphosphate (IP<sub>3</sub>) and [Ca<sup>2+</sup>]<sub>i</sub> as well as cyclic nucleotides. Other groups have studied the effects of ET receptor agonists on both platelet [Ca<sup>2+</sup>]<sub>i</sub> and IP<sub>3</sub>. However, there were no published reports on the effects of ET receptor agonists on platelet cyclic nucleotide levels at the time of this work, and consequently it was considered that this may be a valuable area to investigate. ET receptor mediated effects on cyclic AMP depend on the tissue type or cell type studied. In cultured rat vascular smooth muscle cells ET-1 stimulated cyclic AMP formation (Eguchi, *et al.*, 1993) whereas in the rat tail artery ET decreased cyclic AMP concentrations (Yang, *et al.*, 1991). Although our study did not detect any effect of either ET-1 or SRTX S6c on platelet cyclic AMP levels, an interesting further study might be to examine the effect of ET-1 in the presence of an agent known to increase platelet cyclic AMP levels. Although, due to the putative ET receptor mediated potentiation of adrenaline-induced aggregation, adrenaline may appear an appropriate adenylate

cyclase activator to use, the complex nature of the effects of adrenaline on platelet cyclic AMP, described in chapter 5, may suggest that PGE<sub>1</sub> would be a better adenylate cyclase activator to use in the first instance.

In chapter 6 techniques were used to find supporting evidence for the existence of specific binding sites for ET-1 on platelets in addition to the functional evidence.

First, in chapter 6, ligand binding experiments were described. The techniques were validated by performing binding studies in tissues known to express ET receptors and by performing binding studies with platelets using an  $\alpha$ 2 adrenergic receptor agonist. The binding characteristics of yohimbine to platelet membranes have been previously well documented. In addition, in view of the putative interaction between adrenaline and ET-1 described in the other chapters of this thesis (chapters 3, 4, 7 and 8) it was seen as an appropriate positive control that might allow for further investigation into the possible regulation of  $\alpha$ 2 adrenergic receptors by ET-1. Of the six subjects studied in the platelet/ET binding study, two produced dose dependent displacement of [<sup>125</sup>I]-ET-1 with increasing concentrations of cold ligand, the other four showed no significant binding. The non-specific binding of [<sup>125</sup>I]-ET-1 to the platelet membranes was relatively high; this could not be attributed to the ligand alone since the non-specific binding to the cardiac membranes was approximately half that seen in the platelets. Non-specific binding of [<sup>3</sup>H]-yohimbine to platelet membranes approached zero.

It would, perhaps, have been useful to have had access to non-radioactive I-ET-1 to use in competition with [<sup>125</sup>I]-ET-1 to assess whether this would have resulted in less non-specific binding. Alternatively, another peptide agonist to platelets could have been used as a positive control, such as angiotensin II. As discussed in chapter 6 many of the ligand binding studies with angiotensin II used whole platelet



preparations rather than platelet membranes, as studied here. The two platelet membrane preparations that did show dose-dependent displacement of [ $^{125}$ I]-ET-1 by unlabelled ET-1 gave  $K_D$ s of the same order of magnitude as seen with [ $^{125}$ I]-ET-1 in the rat heart preparations.

In chapter 6 the results of RT-PCR studies were also reported. As in the ligand binding studies this gave inconclusive results because platelet preparations from some individuals did not appear to possess mRNA for ET receptors. Since platelets are anucleate the source of mRNA in platelets is assumed to have come from the parent megakaryocyte. The factors that control the quantity of residual platelet mRNA are not well understood, and the absence of platelet mRNA should not be taken to mean that platelets do not express the receptor. The fact that samples from only some individuals appeared to contain ET receptor mRNA may reflect inter-individual differences in receptor expression but may also reflect differences in other factors, such as RNase activity for example.

*In vitro* work from a few authors (discussed below) has produced evidence for a direct effect of ET-1 on human platelets. However, *ex vivo* studies in animals have suggested that the predominant influence of ET-1 on platelets is indirect and a result of endothelium derived prostanoids. Early studies established interspecies differences in platelet responses to ET-1 (Ohlstein, *et al.*, 1990). In chapter 7 this issue was addressed by comparing the results of *ex vivo* and *in vitro* studies in humans. In addition to assessing the role of the endothelium this study also afforded the opportunity to investigate any effect of other blood cells. *In vivo* exposure to exogenous ET-1 resulted in inhibition of adrenaline, but not ADP induced secondary aggregation. No significant effect was observed on primary aggregation. *In vitro* studies using a concentration of ET-1 estimated to be approximately the same as that achieved *in vivo* (0.1 nM) produced similar results. This study was designed to limit

the effect of endothelium derived prostacyclin, and supported the proposition that ET-1 can act directly on human platelets.

The third aim outlined in chapter 1, to investigate the actions of ET-1 on platelets in diseases where ET-1 is thought to have a pathophysiological role, was addressed in chapter 8. Chapter 8 described two studies; one to examine the effects of ET-1 and SRTX S6c on platelet aggregation and cyclic nucleotide levels in patients with essential hypertension and one to investigate whether altered platelet reactivity to ET is secondary to hypertension or possibly involved in its aetiology. In this latter study a group was studied in whom predisposition to high blood pressure has been defined on the basis of their own blood pressure and the blood pressure of their parents. In the study of essential hypertension ET-1 caused a significant inhibition of secondary platelet aggregation in the control group but not in the hypertensive subjects. However, although there was not a significant difference between the groups in the secondary aggregation to adrenaline alone, it did tend to be higher in the control group and this may have contributed to the significant effect of ET-1 in this group. In addition, subjects with essential hypertension showed a reduced cyclic GMP response to SRTX S6c, compared to the controls. The stimulation of platelet cyclic GMP does not appear to be causally linked to ET-1 inhibition of platelet aggregation, because ET-1 inhibited platelet aggregation in the control group at  $10^{-8}$  M without significantly affecting platelet cyclic GMP levels.

In the study which investigated the effect of ET-1 on platelet aggregation in subjects with a predisposition to hypertension, significant potentiation of primary aggregation only occurred in subjects with blood pressure in the upper tertile and parental blood pressure in the upper tertile. This result suggests that there is a genetic component to increased sensitivity to ET-1 in subjects with a predisposition to hypertension, and

that increased platelet sensitivity to ET-1 may contribute to the development of hypertension.

There were two trends that appeared repeatedly throughout the work described in this thesis. The first was a large inter-individual variation in the effects of ET-1 on platelets. Reproducibility of platelet aggregation studies depend on a number of factors such as concentration of sodium citrate, storage time, temperature and stirring speed. The protocol for aggregation studies was maintained throughout all the studies reported in this Ph.D. project (with the exception of study 2 in chapter 8, which was amended as specified), and hence all conditions both within studies and between studies were constant. Some researchers standardise platelet number for all samples, to reduce variability. However, this was not done in the work of this thesis in order to reflect that variability and not to exclude subjects with a lower platelet number yet still within the normal range.

Marked inter-individual variability within studies (with, presumably, standardised protocols) has been reported for a number of platelet aggregating agents, including vasopressin (Roos *et al.*, 1986), ADP (Arkel, *et al.*, 1977) and adrenaline (Siess, 1989). Furthermore, Vittet reported variability in platelet cyclic AMP regulation and proposed that two separate groups of healthy platelet donors could be distinguished on the basis of adenylate cyclase activity (Vittet *et al.*, 1988). It had been suggested that the variable platelet responses to adrenaline were due to the balance between two adrenaline activated receptors with conflicting actions, the  $\alpha$  and the  $\beta$  adrenergic receptors. However, a heterogeneous response was demonstrated for an  $\alpha_2$  agonist suggesting a more complex explanation (Nieuwland, *et al.*, 1993). Clearly, human platelets from different donors show considerable variability in their responses and, therefore, variability in their responses to ET-1 is not novel. However, from the results of chapter 8 it appears that a possible bimodal distribution

of platelet responses and possibly platelet receptor expression for ET-1 may be in part associated with a genetic factor linked to a predisposition to high blood pressure.

The second trend that was observed in a number of the studies within this thesis was the apparently greater response to SRTX S6c than to ET-1. Although this difference was not statistically significant, it was observed in the aggregation study reported in chapter 4, and the cyclic nucleotide studies in chapter 5 and 9, and should therefore be given some consideration. As discussed above, it does not appear that the ET mediated rise in cyclic GMP is related to the ET mediated inhibition of secondary aggregation. It could, in fact, be related to the ET mediated potentiation of adrenaline-induced primary aggregation. It has been proposed that platelet aggregating agents can stimulate platelet NO synthase and consequently increase platelet cyclic GMP (Radomski, *et al.*, 1990). Although there is no direct evidence for ET-1 stimulation of platelet NO synthase, the report by Radomski establishes that there is a precedent for pro-aggregatory agents stimulating the inhibitory second messenger cyclic GMP. It would appear from the results of chapters 4 and 5 that both the ET-1 mediated potentiation of aggregation and accumulation of cyclic GMP are mediated by the ET<sub>B</sub> receptor and the profiles depicted in figures 4.1 and 5.3 are similar, although the active concentrations of the agonists in the two studies are different. However, although the apparently greater effect of SRTX S6c in potentiating aggregation and causing an accumulation of cyclic GMP may be due to the same mechanism, this is not proven and it is possible that they may be purely coincidental.

The apparently greater effect of SRTX S6c could be mediated at any one of several different stages; availability of agonist to the receptor, multiple receptor involvement or post-receptor regulation. The possibility that differential enzymatic degradation of



the peptides might be responsible was discussed in chapter 5, and does not appear to be a likely explanation for the different magnitude of the effects of ET-1 and SRTX S6c. Another possibility is that the effects are being mediated by more than one receptor. If ET<sub>A</sub> receptor mediated responses were acting in opposition to ET<sub>B</sub> receptor mediated responses, to create an equilibrium, it would be possible that the ET<sub>A</sub> receptor was mediating an inhibition of aggregation and of cyclic GMP accumulation, and thus limiting the observed effect of ET-1 compared to that of SRTX S6c. However, if this were the case one would expect that ET<sub>A</sub> antagonism by BQ-123 would increase the potentiation of aggregation by ET-1. This was not observed when this experiment was performed, as can be seen in figure 4.3. If, alternatively, the proposed ET<sub>C</sub> receptor was a second receptor type present on platelets, and mediating potentiation of aggregation and accumulation of cyclic GMP this would support a greater effect of SRTX S6c. It is difficult, however, to formulate *a priori* reasoning for this latter argument unless an endogenous agonist for the ET<sub>C</sub> receptor, such as ET-3, was involved in the regulation of platelet activity.

The third possibility is that there is different post-receptor modulation of the responses to the two agonists. In 1994 Shrager-Levine demonstrated that sarafotoxins and ETs stimulated cyclic GMP production in the rat cerebellum via the ET<sub>B</sub> receptor (Shrager-Levine *et al.*, 1994). However, the mechanisms responsible for the increase in cyclic GMP were different. ETs stimulated NO production, which resulted in the rise in cyclic GMP, but the increase in cyclic GMP by sarafotoxins was a result of carbon monoxide production. To explain this he proposed that there might be ligand selective coupling of the ET<sub>A</sub> receptor to specific G proteins. To test this in the platelet it would first be necessary to identify at least one of the pathways that result in either the ET<sub>B</sub> mediated accumulation of platelet cyclic GMP or the ET<sub>B</sub> mediated potentiation of aggregation.

Before considering the implications of the work presented in this thesis it would be useful to consider the published results of other groups concerning the effects of ETs and other ET receptor agonists on platelets. In general two types of studies have been performed, *in vivo/ex vivo* and *in vitro*. All of these studies are discussed in some detail in the following paragraphs. The *in vivo* and *ex vivo* studies involved the introduction of the ET receptor agonists, either by bolus or continuous infusion, into whole animals, and consequently the effect of other endothelium derived platelet modulators, such as prostacyclin and NO, are also brought into play. All of these studies were performed in animals and the majority by the same research group. *In vitro* studies have investigated the effect of ET receptor agonists on platelets in the absence of the vascular endothelium, and, consequently, these studies may be better designed to investigate the direct effects of the agonists on platelets. Studies of this type have been performed using platelets from humans and animals.

The *in vivo* and *ex vivo* studies are fewer in number, have generally studied similar parameters, and appear more consistent in their findings. In 1989, three papers were published which concluded that ET-1 and ET-3 inhibited *ex vivo* aggregatory responses in rabbits and dogs, and that this antiaggregatory effect was mediated by prostacyclin (Hermán, *et al.*, 1989; Lidbury, *et al.*, 1989; Thiernemann, *et al.*, 1989). However, Thiernemann also observed a delayed antiaggregatory effect of ET-1 that was not blocked by cyclooxygenase inhibition. The following year Thiernemann published a report on the effect of ET-1 on *in vivo* platelet aggregation in rabbits, where he showed an inhibition of ADP-induced platelet aggregation, by the release of 'an anti-aggregatory cyclooxygenase product' (Thiernemann *et al.*, 1990). Finally, three publications in 1993 suggested that the *ex vivo* anti-aggregatory effect of ET-1 was mediated by the ET<sub>B</sub> receptor (Hermán, *et al.*, 1993; McMurdo *et al.*, 1993a; McMurdo *et al.*, 1993b). All these studies used systemic concentrations

of agonists and, as stated above, were performed in animals. This is in contrast to the study reported in chapter 7 of this thesis which used a locally acting concentration of ET-1 in man.

The publications that have reported *in vitro* studies have examined a variety of parameters in platelets from animals, healthy human subjects, patients with hypertension and patients with chronic renal failure. The results obtained are more disparate than those obtained from the *ex vivo* studies, this appears to be due to a greater variety of experimental conditions. Many of the early studies, those up to 1992, found little evidence for a direct effect of ET-1 on human platelets either alone or in combination with other aggregating agents (Edlund & Wennmalm, 1990; Joseph *et al.*, 1991; Ohlstein, *et al.*, 1990; Patel, *et al.*, 1989). The aggregating agents studied were generally ADP and thrombin, but Ohlstein also studied collagen and adrenaline. However, although Ohlstein and colleagues did not observe any effect in human platelets, they did report a pro-aggregatory effect of ET-1 on ADP-induced aggregation in rabbit platelets indicating inter-species differences in platelet responses to ET-1. In contrast to these other reports, Matsumoto described a pro-aggregatory effect of ET-1 on human platelet aggregation induced by sub threshold concentrations of adrenaline (Matsumoto, *et al.*, 1990). Although not all publications give a complete description of the methods employed, the main difference between the study performed by Matsumoto and the others appears to be the length of time over which the aggregatory response was studied. Matsumoto studied the course of aggregation for 10 min, whereas the others, where specified, used 5 min or less.

In 1992-93 a number of groups reported an inhibitory effect of ET-1 on *in vitro* platelet aggregation and  $[Ca^{2+}]_i$  mobilisation (Astarie-Dequeker, *et al.*, 1992; Takada & Takada, 1992; Touyz & Schiffrin, 1993b). Pietraszek and colleagues proposed that the length of time between the ET challenge and challenge with the aggregating



agent determined whether a pro-aggregatory or an antiaggregatory effect was observed (Pietraszek *et al.*, 1992). He found that ET-1, 2 or 3 could potentiate serotonin responses in platelets when added at or about the same time, but when pre-incubated with the platelets before the addition of serotonin, the ETs inhibited the response. This time-dependent pattern of response is similar to that observed for protein kinase C (Siess, 1989).

In 1995 further work investigated the mechanisms of ET receptor mediated modulation of platelet aggregation. ET-3 inhibited  $[Ca^{2+}]_i$  mobilisation by thrombin and thromboxane  $A_2$  but not by ADP (Astarie-Dequeker, *et al.*, 1995), providing a possible explanation for the absence of ET receptor mediated inhibition of ADP induced secondary aggregation observed in chapters 3 of this thesis. Inhibition of protein kinase C limited the ET-1 inhibition of thrombin-induced aggregation and of the thrombin-induced rise in  $[Ca^{2+}]_i$  (Touyz & Schiffrin, 1995) suggesting that activation of protein kinase C may occur at an early stage of ET-1 stimulation. The mechanism for potentiation of aggregation was investigated by Halim and colleagues, who demonstrated that in single adherent platelets ET-1 induced oscillations in  $[Ca^{2+}]_i$  and in washed platelet preparations ET-1 induced inositol trisphosphate production (Halim *et al.*, 1995). Platelet NO synthase is  $Ca^{2+}$  dependent, and the  $[Ca^{2+}]_i$  oscillations described by Halim and colleagues could provide a mechanism for the increased cyclic GMP observed in chapter 5 of this thesis, which would be consistent with a rise in cyclic GMP being associated with a pro-aggregatory response. However, the precise implications of Halim's study are difficult to discern. Although it demonstrates the ability of ET-1 to evoke a rise in  $[Ca^{2+}]_i$  and  $IP_3$  formation, it does so in platelets treated with aspirin and prostaglandin  $E_1$ . It is not clear, therefore, what role these particular intracellular signalling mechanisms would have in mediating platelet aggregatory responses to ET-1.

Whole blood aggregation studies using ET-1 may involve more complex mechanisms. ET-1 has been shown to have a pro-aggregatory effect on platelets in whole blood due to increased production of platelet activating factor by neutrophils (Lopez-Farre *et al.*, 1991). Hence, the ET-1 potentiation of ADP-induced aggregation described by Knöfler (Knöfler *et al.*, 1995) and the increased potentiation of ADP-induced aggregation observed in patients with chronic renal failure (Heintz *et al.*, 1994) may not be associated with the direct action of ET-1 on platelets, as both these studies were performed in whole blood.

The work presented in this thesis is consistent with the findings of Matsumoto, that ET-1 can potentiate adrenaline-induced platelet aggregation in at least some individuals (Matsumoto, *et al.*, 1990) and has shown that this potentiating effect of ET-1 is dose dependent and mediated by the ET<sub>B</sub> receptor. It also supports the findings of other workers who showed a direct inhibitory action mediated by ET receptors on human platelets and has been able to discriminate between the two opposing effects by studying the functionally and temporally, distinct phases of primary and secondary aggregation. It would appear that ET-1 can modulate platelet aggregation. Plasma concentrations of ET-1, as measured in the study reported in chapter 7, are low at around 2.5 pM, and therefore, it is unlikely that ET-1 acts to regulate basal platelet activity. As the primary function of platelets is to respond to localised areas of damage, it would be more likely for a modulator of platelet activation to have raised levels at or around sites of damage rather than raised systemically.

In 1991 Ohlstein demonstrated that quiescent platelets could stimulate ET-1 synthesis from cultured bovine artery endothelial cells and human umbilical vein endothelial cells (Ohlstein, *et al.*, 1991). It would, therefore, appear possible that the

consequent ET-1 release may act to restrict platelet aggregation. Alternatively, if an increase in plasma ET-1 were to occur coincidentally with increased adrenaline or serotonin, as may occur during ischaemic attack the result may be an increase in platelet aggregation contributing to vasospasm or vessel occlusion.

There is clearly a theoretical role for an agent that might contribute to the formation of platelet aggregates at site of damage but limit the extent of aggregation in order to prevent unnecessary vessel occlusion and excessive release of growth factors. However, if the balance of anti- and pro-aggregatory effects were altered the said agent might contribute to disturbances of blood flow and an increase in shear stress by encouraging repeated platelet aggregation and disaggregation. Alternatively, if the inhibitory mechanisms were absent, as observed in patients with essential hypertension (chapter 8), the same agent might contribute to pathological vasospasm by contributing to the release of platelet derived vasoactive substances such as serotonin and thromboxane A<sub>2</sub>.

The preceding paragraphs speculate on a possible role for ET-1 in platelets in health and disease. However, it requires less speculation when considering the results of chapter 8 to envisage possible clinical implications of the results contained within this thesis. ET receptor mediated responses were altered in subjects with a genetic predisposition to high blood pressure and in patients with established hypertension. In neither of these groups was the effect of adrenaline alone altered suggesting that the effects observed were not simply a generalised increase in the reactivity of platelets due, for example, to higher resting  $[Ca^{2+}]_i$ . In the case of the hypertensive subjects the ET<sub>B</sub> mediated rise in cyclic GMP was attenuated but the pro-aggregatory actions mediated, evidently, by the same receptor type were unaltered. This suggests that there may be multiple signalling pathways linked to a single receptor type and that one or more of these pathways may be altered in essential

hypertension. If the model proposed by Pietraszek (1992), of both anti - and pro-aggregatory effects being mediated by a single receptor type is correct, then the same scenario is evident in the subjects with a genetic pre-disposition to high blood pressure, where a difference was observed in the potentiation of aggregation by ET-1 but not in the inhibitory effect. The obvious question that arises is, are the aberrant ET receptor mediated signalling pathways only involved in platelet activation or are they important intracellular mechanisms in other cells, such as vascular smooth muscle cells ?

Before extrapolating from the platelet to the whole vascular system, further work remains to be carried out in order to completely characterise the effects of ET-1 on human platelets. The receptor which mediates the anti-aggregatory actions of ET-1 *in vitro* remains to be characterised. Both Astarie-Dequeker and Pietraszek suggested equal efficacy of all three ET peptides for the inhibitory actions, this would imply that the effect is ET<sub>B</sub> mediated (Astarie-Dequeker, *et al.*, 1992; Pietraszek, *et al.*, 1992). However, there do not appear to be any published studies reporting the effects of selective antagonists. There also remains the findings of Touyz of a BQ-123 sensitive pH effect of ET-1 in human platelets (Touyz & Schiffrin, 1993b).

The precise second messengers mediating the effects of the ETs are not been well established. Although a number of groups have produced evidence for ET receptor agonists altering second messenger systems (including chapter 5 of this thesis), only Touyz has demonstrated inhibition of a specific intraplatelet pathway and an attenuation of the effect of ET-1 on platelet aggregation pharmacologically (Touyz & Schiffrin, 1995). Even in these experiments the effects of ET-1 were not completely abolished by protein kinase C inhibition. It is not known whether protein kinase C is activated directly or as a result of the formation of diacyl glycerol, or which isoform of the kinase is involved. There are a number of protein kinase C

enzymes in platelets (Wang *et al.*, 1993), and the identification of the precise enzyme and the evaluation of its translocation would help considerably in elucidating the intracellular mechanism for ET-1 in platelets.

In conclusion, the work presented in this thesis was designed to test the hypotheses set out in chapter 1. These hypotheses were that; ET-1 can both potentiate and inhibit platelet aggregation by acting on specific platelet surface receptors and that the pro-aggregatory and inhibitory effects are mediated by different ET receptors, furthermore that in essential hypertension the action of ET-1 on platelets may relate to the pro-aggregatory state of platelets in this condition. ET-1 was shown to modulate platelet aggregation in a biphasic manner. The potentiation was shown to be mediated by the ET<sub>B</sub> receptor. However, the receptor which mediated the inhibitory actions of ET-1 was not characterised, and hence it was not established whether the opposing actions are mediated by different receptors or not. In essential hypertension the ET-1 mediated inhibition of aggregation observed in normal controls was absent, consequently the altered response to ET-1 on platelets may relate to the pro-aggregatory state of platelets in hypertension. Furthermore, the potentiation of platelet aggregation by ET-1 may be associated with a genetic predisposition to high blood pressure.

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# Endothelin-1 and Aggregation of Human Platelets In Vitro

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**Summary:** Endothelin-1 (ET-1) is a potent vasoconstrictor peptide produced by endothelial cells. We investigated whether ET-1, like other potent endothelium-derived vasoactive agents, interacts directly with human platelets in vitro. Platelet-rich plasma was obtained from healthy male volunteers and incubated with ET-1 (1  $\mu$ M) or vehicle (sodium chloride 154 mM) for 10 min at 37°C. Platelet aggregation was measured by the Born method, using light transmittance through the plasma sample as an index of activation. Although a significant increase in light transmittance was observed when plasma was incubated with ET-1 compared with vehicle, ( $3.8 \pm 0.4\%$  versus  $2.7 \pm 0.2\%$ ;  $n = 24$ ;  $p = 0.038$ ), this effect was small and is

unlikely to be of biologic significance. To investigate the possibility that ET-1-stimulated platelet nitric oxide (NO) synthesis might be masking a direct aggregatory effect of ET-1, in a second study in six subjects N<sup>G</sup>-monomethyl-L-arginine (L-NMMA, 10 and 100  $\mu$ M), an inhibitor of NO synthase, was preincubated with the plasma before the addition of ET-1 (1 nM and 1  $\mu$ M). No significant difference was observed whether samples were incubated with L-NMMA alone or with L-NMMA and ET-1. The results of this study suggest that ET-1 does not have a major direct effect as a platelet aggregating agent. **Key Words:** Human—Platelets—Endothelin-1—Nitric oxide.

Endothelin-1 (ET-1) is a potent 21-amino acid vasoconstrictor peptide produced by endothelial cells (1). Bolus administration of ET-1 causes transient vasodilation (2) followed by prolonged vasoconstriction (1). Whereas the prolonged vasoconstrictor effects of ET-1 are paracrine and are believed to be mediated by an ET<sub>A</sub> receptor subtype situated on vascular smooth muscle (3), the brief vasodilator effects appear to be autocrine and mediated by an ET<sub>B</sub> receptor situated on endothelial cells (4). Vasodilation can be caused by production of either nitric oxide (NO), prostacyclin (PGI<sub>2</sub>), or a combination of the two, depending on the species and the vascular bed studied (5,6).

Previous studies have shown that ET-1 inhibits platelet aggregation in vivo and ex vivo (7,8). As this is associated with a rise in platelet cAMP, it has been attributed to endothelial PGI<sub>2</sub> production, although the effect is only partially attenuated by indomethacin (7). We examined the direct effects of ET-1 on human platelets in vitro in the absence of the influence of other endothelial factors, using Born aggregometry. To investigate whether platelet aggregation induced by ET-1 is masked by stimulation of platelet NO synthase, we also performed experiments in the presence and absence of the NO

synthase inhibitor N<sup>G</sup>-monomethyl-L-arginine (L-NMMA).

## METHODS

Subjects were healthy men aged 21 to 44 years ( $n = 24$ ). None of the participants had taken vasoactive drugs or drugs known to alter platelet activity, including nonsteroidal anti-inflammatory drugs, in the preceding 10 days. All subjects had abstained from tobacco and alcohol for a minimum of 2 h, and rested supine for 30 min before venesection.

The aggregation protocol was based on that described by Gow et al. (9). Venous blood (54 ml) was drawn through a 19 SWG steel needle into a polypropylene syringe and immediately transferred into six 10-ml tubes, each containing 1 ml acid citrate dextrose (8 mg/ml citric acid, 22 mg/ml sodium citrate, 20 mg/ml glucose). Blood was centrifuged at 110  $g$  for 10 min at room temperature to obtain platelet-rich plasma (PRP). PRP was removed and the residue was spun for a further 20 min at 3,000  $g$  to obtain platelet-poor plasma (PPP). PRP was stored under 5% CO<sub>2</sub>:95% O<sub>2</sub> in sealed polypropylene tubes at room temperature until required. Aggregation was assessed at 37°C on a six-channel aggregometer (Malin) linked to a Macintosh computer through a MacLab digital analogue converter.

Experiments were performed according to the method

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of Born (10). Briefly, the extent of aggregation was calculated by the change in light transmittance, where light transmittance through PRP was taken as 0% aggregation and that through PPP as 100% aggregation. Aggregometer recordings were examined for the three classical signs of platelet activation and aggregation: shape change, primary aggregation, and secondary irreversible aggregation.

In the first study in 24 subjects, 900  $\mu$ l of sample was incubated for 3 min before the addition of 50  $\mu$ l vehicle (sodium chloride 154 mM), followed 1 min later by 50  $\mu$ l ET-1 (final concentration 1  $\mu$ M) or vehicle. In the second study in six subjects, 900  $\mu$ l of sample was incubated for 3 min before the addition of 50  $\mu$ l L-NMMA, (final concentrations 10 and 100  $\mu$ M) or vehicle, followed 1 min later by the addition of 50  $\mu$ l ET-1 (final concentrations 1 nM and 1  $\mu$ M) or vehicle. Responses were followed for 10 min after the addition of ET-1. Both L-NMMA (Sigma Chemical Co. Ltd, Poole, U.K.) and ET-1 (NovaBiochem, Nottingham, U.K.) were dissolved in sodium chloride 154 mM. Results are expressed as means  $\pm$  SEM. Statistical analysis was by analysis of variance followed by Student's paired *t* test where applicable, with a value of *p*  $\leq$  0.05 accepted as significant.

## RESULTS

Aggregation was slightly but significantly greater in the 10-min period after the addition of ET-1 than after saline ( $3.8 \pm 0.4\%$  and  $2.7 \pm 0.2\%$ , respectively; *n* = 24; *p* = 0.038). There were no significant differences in the responses to L-NMMA whether or not ET-1 was present, showing that L-NMMA did not unmask aggregation to ET-1 (Table 1). No shape change or secondary aggregation was observed in any of these studies.

## DISCUSSION

A slight but significant difference in aggregation, as observed by light transmittance, was noted between the control sample and the sample incubated with ET-1 (1  $\mu$ M) in the first study. Such a difference is unlikely to be of biological significance and may not be related to platelet aggregation. It is unlikely that a substantial aggregatory effect would emerge with higher ET-1 concentrations because the concentration employed was substantially higher than the  $K_d$  for either the ET<sub>A</sub> or ET<sub>B</sub> receptor (3,4), which is in the nanomolar range, and higher than concentrations associated with biologic activity (5).

TABLE 1. Aggregation, as measured by change in light transmittance, from coincubation of L-NMMA with ET-1 or vehicle (*n* = 6)

	Vehicle	ET-1 (1 nM)	ET-1 (1 $\mu$ M)
Vehicle	$2.3 \pm 0.29\%$	—	—
L-NMMA 10 $\mu$ M	$3.2 \pm 0.42\%$	$3.0 \pm 0.85\%$	$2.8 \pm 0.79\%$
L-NMMA 100 $\mu$ M	$3.3 \pm 0.31\%$	$3.8 \pm 0.53\%$	$2.8 \pm 0.77\%$

From these data it can be concluded that ET-1 has, at most, very limited aggregating properties and is unlikely to act directly as an aggregating agent at physiologic concentrations.

Light transmittance after incubation of platelets with L-NMMA at concentrations of 10 and 100  $\mu$ M was not significantly different in the presence or absence of ET-1 at either 1 nM or 1  $\mu$ M. This result excludes the possibility that ET<sub>B</sub>-mediated stimulation of NO generation masks a direct aggregatory effect of ET-1.

Although ET-1 may not stimulate platelet aggregation directly, there is evidence that it modulates platelet aggregation initiated by other agonists. ET-1 can modify epinephrine- (11,12) and thrombin-induced aggregation (13). Dequeker et al. (13) also demonstrated that endothelin-3 produces a small but significant decrease in  $Ca^{2+}$  mobilization when coincubated with thrombin. Stimulation of the ET<sub>A</sub> receptor might be expected to mediate phosphatidylinositol hydrolysis, resulting in platelet aggregation and a concomitant rise in intracellular calcium [ $Ca^{2+}$ ]<sub>i</sub>. By activating NO synthase, stimulation of the ET<sub>B</sub> receptor might reduce [ $Ca^{2+}$ ]<sub>i</sub> and inhibit platelet aggregation. ET-1 may act like other potent vasoactive agents, angiotensin II (14) and epinephrine (15), via specific membrane-bound receptors to modify the actions of other aggregating agents without directly initiating platelet aggregation. This requires further investigation.

From our results, we conclude that ET-1 causes significant but not physiologically relevant platelet aggregation and that aggregatory effects are not masked by ET<sub>B</sub>-mediated NO production.

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